Universidade de Aveiro Departamento de Química

2019

ANA PATRÍCIA PINHEIRO VIEIRA

ESTUDO DA CONSERVAÇÃO E CARACTERIZAÇÃO DE UM IOGURTE FERMENTADO SOB PRESSÃO

STORAGE STUDY AND CHARACTERIZATION OF AN YOGHURT FERMENTED UNDER PRESSURE

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia (ramo Alimentar), realizada sob a orientação científica do Doutor Jorge Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

“Certifica-te que a criança que outrora foste teria orgulho do adulto em que te tornaste!”

Poucotrintona, 2016

o júri

presidente

Doutor José António Lopes da Silva
professor auxiliar do Departamento de Química da Universidade de Aveiro

vogais

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palavras-chave Fermentação, iogurte, conservação, pressão, bactérias acido-láticas;

resumo

Este trabalho teve como objetivo estudar o armazenamento sob refrigeração (4°C por 23 dias) de iogurte produzido a 43°C sob pressões sub letais, a 10, 20, 30 e 40 MPa, em comparação com o processo de fermentação à pressão atmosférica (0.1 MPa). A contagem de bactérias lácticas *(Streptococcus thermophilus* e *Lactobacillus delbrueckii spp. Bulgaricus*, LAB) e parâmetros de qualidade como pH, acidez titulável, sinérese e cor foram avaliados, juntamente com análise textural para inferir como a pressão impactaria no iogurte obtido ao longo do armazenamento. Além disso, foi realizada uma impressão digital de metabolitos por ressonância magnética nuclear (RMN), açúcares e ácidos orgânicos por cromatografia líquida de alta eficiência (HPLC), determinação e quantificação de ácidos gordos totais (TFA) por cromatografia de fase gasosa com detetor de ionização de chama (GC-FID) e determinação dos principais compostos voláteis por cromatografia de fase gasosa acoplada a espetrometria de massa por ionização de eletrões (GC-EI-MS).

Maiores pressões de fermentação resultaram em cargas LAB ligeiramente menores (um máximo de diferença de 1.01 Log (UFC/mL)) e originou um aumento do tempo de fermentação (máximo de 3h 25 min), sinérese (máximo de 44 %), todos para iogurtes fermentados a 40 MPa e firmeza (máximo de 2.5 vezes) para 30 MPa. Sob refrigeração, notou-se que as LAB estavam mais ativas durante os primeiros 15 dias de armazenamento em iogurtes fermentados sob pressão (cargas crescentes até 0.54 Log (UFC / mL)).

Os parâmetros cor, pH e acidez titulável não foram afetados pela pressão ou armazenamento.

As análises metabólicas obtidas por RMN permitiram verificar que apenas os compostos 2,3-butanediol, acetoína, diacetil e formato variam com o aumento da pressão e, provavelmente, as enzimas diacetil redutase, redutase acetoína e acetolactato descarboxilase são ativadas com o aumento de pressão. A pressão também afetou o consumo de lactose e o teor de TFA. Os iogurtes fermentados com a 40 MPa apresentaram menor teor de lactose (39,7 % da redução total de açúcar) e menor teor de TFA (12301.5 µg/g de iogurte, 56,1 % menos que o fermentado a 0.1 MPa). Assim, o consumo destes iogurtes (fermentados sob pressão) poderá ser uma alternativa saudável para o consumidor, graças ao seu baixo teor em açúcares e gorduras, mas também os índices de qualidade lipídica obtidos, demonstraram-se interessantes, podendo contribuir para uma dieta saudável.

O método aplicado para determinar compostos voláteis permitiu identificar 131 compostos e alguns deles só apareceram nos iogurtes fermentados sob pressões mais elevadas, podendo ser característicos dos mesmos.

Finalmente, e apesar do baixo número de participantes na análise sensorial, é importante destacar a maior aceitabilidade e preferência do iogurte fermentado sob pressão, principalmente a 10 e 20 MPa.

Mais pesquisas são de interesse para averiguar o potencial biotecnológico dos processos de fermentação sob alta pressão sub-letal em geral e, em particular, para a produção de iogurte.

keywords

Fermentation, yoghurt, storage, pressure, lactic acid bacteria;

Abstract

This work aimed to study refrigeration storage (4°C for 23 days) of yoghurt produced at 43°C under sub-lethal high pressure, at 10, 20, 30 and 40 MPa, in comparison with the fermentation process at atmospheric pressure (0.1 MPa). Lactic acid bacteria (*Streptococcus thermophilus* e *Lactobacillus delbrueckii spp. Bulgaricus*, LAB) and quality parameters like pH, titratable acidity, syneresis and colour were evaluated, along with sensorial and textural analyses to infer how pressure would impact the obtained yoghurt along storage. Moreover, it was done a metabolite fingerprinting by nuclear magnetic resonance (NMR), sugars and organic acids assessment by high performance liquid chromatography (HPLC), total fatty acids (TFA) determination and quantification by gas chromatography with a flame ionization detector (GC-FID) and determination of the principal volatile compounds by gas chromatography electron ionisation mass spectrometry (GC-EI-MS).

Higher fermentation pressures resulted in slightly lower LAB loads (a maximum of 1.01 Log (CFU/mL)) and increased the fermentation time (a maximum of 3h 25 min), syneresis (a maximum of 44 %), all for 40 MPa and firmness (a maximum of 2.5-fold) for 30 MPa. Under refrigeration, LAB were more active during the first 15 days of storage in yoghurts fermented under pressure (increasing loads up to 0.54 Log (CFU/mL)). Colour, pH and titratable acidity parameters were not affected by pressure or storage.

Metabolomic analyses by NMR permitted to verify that just 2,3-butanediol, acetoin, diacetyl and formate vary with the increase of pressure and probably pressure active diacetyl reductase, acetoin reductase and acetolactate decarboxylase. Pressure also affect lactose consumption and the content of total fatty acids. Yoghurts fermented at 40 MPa had the less content in lactose (39.7 % of total sugar reduction) and the less content in TFA (12301.5 µg/g of yoghurt, 56.1 % less than the fermented under 0.1 MPa). So, the consumption of these yoghurts (fermented under pressure) can be a good consumer choice because of their low content in sugars and fats, but also the lipid quality indices obtained were very good and may contribute to healthy diet.

The method applied to determine volatile compounds allowed to identify 131 compounds and some of them were only found in the yoghurts fermented under higher pressures and may be characteristic of them.

Finally, and despite of the low number of participants in the sensory analysis, it is important to highlight the higher acceptability and preference of the yoghurt fermented under pressure, mainly at 10 and 20 MPa.

Further research is of interest to ascertain the biotechnological potential of fermentation processes under sub-lethal high pressure in general and in particular for yoghurt production.

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Abbreviatures

A	*Streptococcus thermophilus* with acidifying proprieties
ANOVA	two-way Analysis of Variance
CFU	Colony-forming units
CHD	Coronary heart diseases
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
EMP	Embden-Meyerhoff-Parnas pathway (Glycolysis)
EPS	Exopolysaccharides
ESB	Escola Superior de Biotecnologia
FA	Fatty acid
n-3	Fatty acids omega 3
n-6	Fatty acids omega 6
FAME	Methyl esters of fatty acids
FDA	Food and Drug Administration
FDR	False Discovery Rate
FFA	Free fatty acids
G'	Elastic modulus
G''	Viscous modulus
GC-FID	Gas Chromatography with a flame ionization detector
GC-EI-MS	Gas chromatography electron ionisation mass spectrometry
GC-MS	Gas Chromatography - Mass Spectrometry
GRAS	Generally recognized as safe
HDL	High density lipoprotein
HP	High pressure
HPLC	High performance liquid chromatography
HSD	Tukey's Honestly Significant Differences
IA	Index of atherogenicity
IT	Index of thrombogenetic
L. bulgaricus	*Lactobacillus delbrueckii ssp. bulgaricus*
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein

LOD	Limit of detection
LOQ	Limit of quantification
m/m	Mass/mass
MeOH	Metanol
MUFA	Monounsaturated fatty acid
MW	Molecular Weight
N	Newton
NMR	Nuclear magnetic resonance
OH	Ohmic heating
PA/PE	Polyamide/polyethylene
PC	Principal Component
PCA	Principal Component Analysis
PEF	Pulsed electric fields
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase system
PUFA	Polyunsaturated fatty acid
R	*S. thermophilus* with ropy proprieties
RDI	Required Daily Intake
S. thermophilus	*Streptococcus thermophilus*
SFA	Saturated fatty acid
SNF	Solids-non-fat
SNR	Signal-to-noise ratio
SPME	Solid-phase microextraction
TA	Titratable acidity
TFA	Total fatty acid
TSP-*d*4	3-(trimethylsilyl)propionic-2,2,3,3-*d*4 acid, sodium salt
UCP	Universidade Católica do Porto
US	Ultrasound
USFA	Unsaturated fatty acids
v/v	Volume/volume
VFA	Volatile fatty acids
w/v	Weight/volume
w/w	Weight/weight
β-gal	β-galactosidase
β-Pgal	β-phosphogalactosidase

CHAPTER I – STATE OF ART

THIS SECTION COMPRISES AN EXTENSIVE, BRIEFLY COMPILED LITERATURE REVIEW REGARDING YOGHURT PRODUCTION, ITS CHARACTERISTICS AND FERMENTATION AT NON-CONVENTIONAL CONDITIONS

1. Yoghurt

1.1. Introduction

The true origin of fermented milks is difficult to establish but it is suggested that they originated in western Asia and were carried to the east, where new variants were developed to suit the different climate **(McKevith and Shortt, 2003)**. Fermentation was one of the first methods used for milk preservation, resulting in products with an extended shelf life due to its low pH **(Tamime and Robinson, 1999)**. Cheese and yoghurt are the most popular dairy products derived from fermented milk **(Hill and Kethireddipalli, 2013)** and the word "yoghurt" is derived from the Turkish word "*jugurt*" but different names are used in different countries **(Tamime and Deeth, 1980)**.

Yoghurt is a semi-solid fermented milk product and is defined by the Food and Drug Administration (FDA) as a fermented dairy product derived from the fermentation of milk by two species of bacterial cultures, *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii ssp. bulgaricus* (*L. bulgaricus*) **(Freitas, 2017)**. So, these bacteria are starter cultures for yoghurt production and use nutrients in milk to support their growth to produce lactate, that reduces milk pH, inhibit the development of many pathogenic and spoilage microorganisms **(Hill and Kethireddipalli, 2013; McKevith and Shortt, 2003; Tamime and Robinson, 1999)**.

In many countries (e.g., Sweden, France, Belgium, Portugal, Spain, United States of America), the current legislation only allows *L. bulgaricus* and *S. thermophilus* to be used to produce yoghurt. For example, in case of United Kingdom, the yoghurt can be made using one or both strains. In other countries, like Switzerland, species such as *L. acidophilus* and *Bifidobacteria* spp. may be used in addition to the traditional yoghurt strains. It is likely that the new Codex Alimentarius (for milk and milk products) standard will build on the draft definition currently used for mild yoghurt (a product made from *S. thermophilus* and *Lactobacilli* other than *L. bulgaricus*), but in other countries like Japan and Finland, there are other requirements for yoghurt production **(McKevith and Shortt, 2003)**.

The increase in the popularity of yoghurt in recent decades has been attributed to its health benefits and the wide diversity of flavours, compositions, and viscosities available to consumers (**Boylston, 2012; Hill and Kethireddipalli, 2013; Tamime and Deeth, 1980**). In 2015, it a study was performed by Marktest Target Group Index, concluding that about

5.3 million Portuguese consume liquid yoghurt, and women have higher rates of consumption than men (66.8 % and 56.6 %, respectively), while among the ages, it is among the target group of 35 to 44 years that there is a greater propensity to consume these yoghurts **(Marktest, 2016)**.

1.2. Yoghurt industrial production

The production of yoghurt starts with the proper selection of raw materials and accurate formulation to produce consistent quality of a liquid mix for a yoghurt under production **(Chandan, 2017; Tamime and Robinson, 1999)**. Most of the industrialized yoghurt productions uses cow's milk, although, goat milk, water buffalo and sheep's milk, are also used **(Hill and Kethireddipalli, 2013; Tamime and Deeth, 1980)**. The typical gross composition (g/100 g) of these milk types is described in **Table 1**.

Table 1 - Typical cross composition (g/100 g) of cow, sheep, water buffalo and goat milk. Adapted from **Hill and Kethireddipalli (2013).**

Composition (g/100 g)	Cow	Dairy Sheep	Water Buffalo	Goat
Fat	3.9	7.2	7.4	4.5
Total protein	3.2	4.6	3.8	3.2
Casein	2.6	3.9	3.2	2.6
Whey	0.6	0.7	0.6	0.6
Lactose	4.6	4.8	4.8	4.3
Ash	0.7	0.9	0.8	0.8
Total solids	12.4	17.5	16.83	12.8

When refrigerated milk arrives at the yoghurt industry/plant, the first step is to modify its composition. This process involves clarification of milk into cream and skim milk, followed by standardization to whole, partially skimmed or skimmed milk, or even creaming to the desired fat and solids-not-fat (SNF) content **(Boylston, 2012; Tamime and Deeth, 1980**). While the average fat content of milk ranges from 3.7 to 4.2 % (w/w), the fat content of commercial yoghurts is approximately 1.5 % for medium fat yoghurt and 0.5 % for low fat yoghurt (**Boylston, 2012; Tamime and Deeth, 1980; Tamime and Robinson, 1999**). The percentage of SNF in the case of existing legal standards, ranges from 8.2 to 8.6 g/100 g of yoghurt **(Tamime and Robinson, 1999)**. To increase the SNF content, the industry,

most commonly adds powder or skim milk to the yoghurt mixture, but many other ingredients may also be used as seen in **Table 2**. Added solids improve yoghurt viscosity and consistency, reduce syneresis, and impart a better mouth-feel, besides contributing to specific functionalities related to the material added. The yoghurt milk base is usually also fortified with non-milk solids such as stabilizers/emulsifiers, and sweetening agents (flavours and fruit preparations) **(Tamime and Robinson, 2007)**.

Table 2 - Characterization of the ingredients added during the standardization and fortification of the base milk to produce yoghurt. Adapted from **Hill and Kethireddipalli, (2013), Simpson *et al.* (2012)** and **Tamime and Robinson, (1999).**

Ingredients added	Examples	Function
Solids-non-fat (SNF)	Milk powder or skimmed (3 – 4 % is recommended); concentrated milk, high-protein milk powders, buttermilk powder, whey powder/concentrate, casein powder, and even non-milk proteins (e.g. proteins from soy, legumes, or sweet potato)	Improve the body and decrease the size of the fat globule
Solids	E.g. phospholipids from buttermilk possess emulsifying properties	Improve yoghurt viscosity, consistency, reduce syneresis, and impart a better mouth-feel
Stabilizers	Natural: gelatin (225/250 Bloom), pectin, guar and locust bean gums, cereal starches, alginates and K-carrageenan; Modified natural/semi-synthetic gums: carboxymethyl cellulose, xanthan gum, low-methoxy pectin, and modified starches; hydrocolloids	Increase the firmness and viscosity of yoghurt and minimize syneresis through their ability to bind with water and the milk proteins to stabilize the protein network
Sweeteners	Sugars: sucrose, invert sugar, glucose, and fructose; Non-caloric high-intensity sweeteners: aspartame and saccharin	Increase the sweetness of yoghurt

Following standardization and fortification of the base milk with a cocktail of milk and non-milk solids, the mixture is pasteurized with typical conditions, for example during 30 minutes at 85°C. Pasteurization achieves not only the inactivation of pathogenic and spoilage vegetative microbes, but also reduces the oxygen, nitrogen and carbon dioxide in the milk to provide a good growth media for the starter cultures, and, most importantly, bring favourable changes in the physicochemical properties of milk.

The pasteurized mix is then homogenized, usually by a single-stage homogenizer at pressures ranging from 15 to 20 MPa and this step is very important in manufacturing yoghurt from milk containing fat, because it will split the fat globules into smaller globules which become coated with a new membrane comprised largely of casein submicelles.

Following heat treatment, the milk is cooled to the incubation temperature (40 – 45°C) and pumped into jacketed fermentation tanks. The starter culture, lactic acid bacteria (LAB), (≈ 3 % w/w of *S. thermophilus* and *L. bulgaricus* in a 1:1 ratio) is directly added to the fermentation tanks. LABs have a synergistic effect on each other's growth and should be present in approximately equal numbers for optimal flavour development. The actual fermentation can take place either in retail containers (set-style yoghurt) or in bulk tanks (stirred yoghurt). Temperatures typically ranging from 37–43°C under quiescent conditions and pH and/or titratable acidity (TA) are carefully monitored during this period. When pH reaches 4.6 (TA of 0.85 – 0.90 %), i.e. when a continuous solid mass of gel is formed, the yoghurt is immediately cooled to nearly 5°C, being afterwards stored, or furtherly processed to produce other yoghurt forms. Cooling is a critical step in yoghurt production and the objective is to reduce the metabolic activity of the starter culture and hence, to control the acidity of the yoghurt. The cooling rate needs to be carefully controlled so that the final product has the desired level of acidity and gel structure, because, while slow cooling can increase yoghurt acidity, very rapid cooling causes whey separation, possibly due to excessive contraction of the protein matrix **(Hill and Kethireddipalli, 2013)**.

Various flavouring agents (fruits, natural and/or synthetic flavours) can be added to yoghurt. In set-style yoghurt, these are normally added to the mix before incubation, but in the stirred type they are often incorporated into the formed gel. The final product needs to be storage at 5°C or less, to protect its characteristics and limit the growth of yoghurt starters. A global view of the process of yoghurt production is represented in **Figure 1** (**section 1.3**).

1.3. Yoghurt Types

Yoghurt manufacturing methods, raw materials and formulation vary widely from country to country, resulting in products with a diversity of flavour and texture characteristics (**Boylston, 2012**). Relatively to different forms of yoghurt, the most common are the set-style and stirred forms. Set-style yoghurt is fermented and packed (retail

container), with colour and flavours added to the container prior to the addition of the inoculated milk and is characterized by a firm, gel-like structure. Stirred-style yoghurt is fermented in a vat before packing and, in this phase, flavours and colourants are added. The gel structure is broken before cooling and packaging. In this type of yoghurt, extensive amounts of syneresis (expelling of interstitial liquid due to association of the protein molecules and shrinkage of a gel network, that increase with the incubation temperature), resulting in a thinner product. Furthermore, liquid yoghurt is also popular and may be considered as stirred yoghurt of low viscosity (**Figure 1**). In fact, there are much more yoghurt types whose characteristics are dependent of the production process and bacterial supplementation (e.g. probiotic supplementation to obtain probiotic yoghurt). Some of these types are also represented in **Figure 1**.

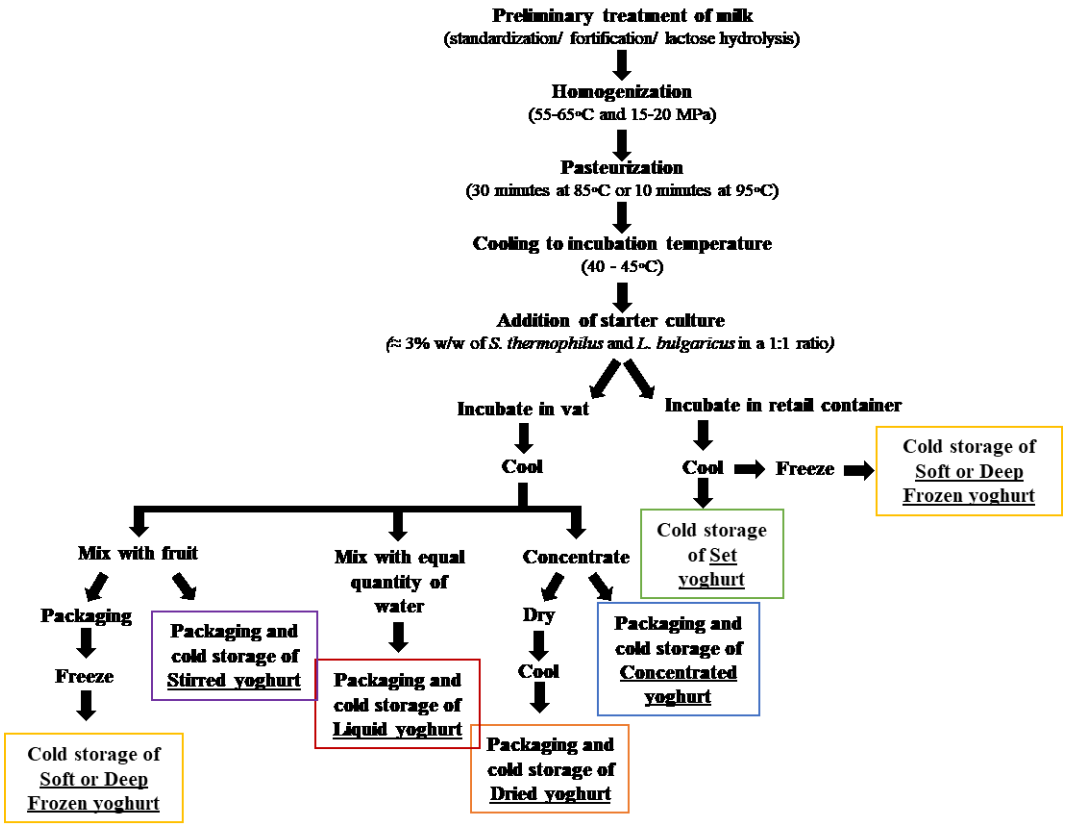


Figure 1 - Industrial production of the main types of yoghurt. Adapted from **Hill and Kethireddipalli, (2013), Tamime and Robinson, (1999)** and **Tamime and Deeth, (1980)**.

Therefore, there is a lot of different types of yoghurts and all of these may have different fat (fat, medium or low) content, nutrient contents and may contain added flavours

or not (plain/natural, fruit or flavoured) or colours. Note that the term *plain yoghurt* means that no additive was added after the yoghurt fermentation. In **Table 3** is represented a typical composition of a plain yoghurt.

Table 3 - Typical composition of plain yoghurt products **(Chandan, 2017)**.

Yoghurt Style		Milk fat (%)	Milk Solids-Non-Fat (%)	Sugar solids (%)	Stabilizer (%)
Plain	Whole milk	3.25 - 3.5	11.0 - 12.0	0	0 - 1.0
	Low fat	1.0	14.2	0	0-0.75
	Non-fat	0.3 - 0.5	14.0 - 15.0	0	0 - 0.75

1.4. Yoghurt Starter Cultures

Fermentation is a biological process and in the context of cultured dairy products, the agents of fermentation are microorganisms **(Vedamuthu, 2006)**. Most cultures present in yoghurt belong to the group of microorganisms known as LAB because they use lactose, the naturally occurring milk sugar, and metabolize it into lactic acid. LAB have been used for food preservation by fermentation for thousand years **(Freitas, 2017)** and tend to be generally recognized as safe (GRAS), although some strains are pathogenic (**Bamforth, 2005**). The LAB used in the development of cultured dairy products include *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Lactobacillus* genera **(Tamime and Deeth, 1980)**.

According to the Codex Alimentarius for milk and milk products, yoghurt should to have at least 10^7 colony-forming units per gram (CFU/g) in total of *L. bulgaricus* and *S. thermophilus* (**Codex Alimentarius International Food Standards, 2003**). Although, other cultures may be added to yoghurt even though they are not required **(Hill and Kethireddipalli, 2013)**, this Codex was accepted by several countries, including Portugal.

The metabolism of the yoghurt cultures contributes to the texture, characteristic flavour and composition of yoghurt. Apart from its nutritional properties, the primary starter function is to generate lactic acid by the fermentation of the major sugar in milk, lactose. For this reason, a biggest advantage for conventional yoghurt is to help with lactose digestion for lactose intolerants because of the presence of lactase enzyme in LAB **(Freitas, 2017; Vedamuthu, 2006)**. In this section, it will be addressed the general metabolism of *S.*

thermophilus and *L. bulgaricus* to better understand their fermentation and composition of final product.

1.4.1. Lactic Acid Bacteria Symbiotic Relations

L. bulgaricus and *S. thermophilus* are thermophilic, gram-positive bacteria **(Boylston, 2012**), facultative anaerobic, non-motile and non-spore-forming bacteria, but have different aspects. The morphology of the first mentioned is rods with rounded ends shape and the second is cocci (ovoid shape with irregular segments), both have similar growth temperature and the same primary metabolic products (lactate and acetaldehyde), but in terms of content, *L. bulgaricus* produce more lactic acid than *S. thermophilus* (**Boylston, 2012**). **Table 4** summarizes the growth characteristics of the two specific LAB that produce yoghurt.

Table 4 - Characteristics of lactic acid bacteria used in yoghurt production with representation of morphology stained cells under light microscope. Adapted from **Simpson *et al.* (2012)** and **Vedamuthu, (2006).**

Lactic Acid Bacteria (LAB) (yoghurt starter culture)	**Morphology**	**Lactose Fermentation**	**Temperature for grow (◦C)**			**Lactic Acid Production (%)**
			Minimum	Optimal	Maximum	
S. thermophilus	Cocci 	Homofermentative	20	40 - 45	50	0.9
L. bulgaricus	Rods 	Homofermentative	22	40 - 45	52	2.5

In milk, *L. bulgaricus* and *S. thermophilus* coexist and interact beneficially in a stable associative relationship also known as protocooperation (also known as biochemical mutualism, involving the exchange of metabolites and/or stimulatory factors) **(Liu *et al.*, 2009)**, meaning that these bacteria have a synergistic effect on each other. It is known that *L. bulgaricus* stimulates *S. thermophilus* by providing essential growth requirements. Glycine, valine, isoleucine, cystine, aspartic acid, glutamic acid, tryptophane and histidine are the essential amino acids for *S. thermophilus* growth. The absence of some of these amino

acids reduces the growth of this bacterium. Furthermore, peptides that contain lysine also stimulate their grow, on the other hand, *S. thermophilus*, under anaerobic conditions, produces stimulatory substances for *L. bulgaricus* such as formic and pyruvic acid **(Tamime and Deeth, 1980)**. More information concerning the symbiotic relationship between these two LAB is provided in **section 1.4.4.2.**

1.4.2. Metabolism of Carbohydrates

Energy is required by microorganisms to maintain their life cycle, and such energy can be provided to the bacterial cell via different systems. LAB do not possess the cytochrome system for electron transport or enzymes to operate the anaplerotic pathways and tricarboxylic acid cycle, the energy can only be supplied by the fermentation of carbohydrates (sugars) **(Tamime and Deeth, 1980; Tamime and Robinson, 1999)**. Such bacteria are only weakly proteolytic and lipolytic, which means that they are quite 'mild' with respect to their tendency to produce pungent flavours **(Bamforth, 2005)**.

Homofermentative LAB ferment sugars, namely lactose and the key step in this metabolic pathway is at the entry of lactose into the cell. Lactose can enter into the cell by two systems: **i)** Lactose is phosphorylated by phosphoenolpyruvate (PEP) during translocation by PEP-dependent phosphotransferase system (PTS). This mechanism is known as PEP:PTS and has four proteins involved in translocation of lactose from outside to the inside of the cytoplasmic membrane as lactose-6-phosphate **(Marshall and Tamime, 1997)**. **ii)** Lactose is transported by cytoplasmatic proteins (permeases) that translocate it without chemical modifications.

In the first system, lactose-6-phosphate is hydrolysed, within the microorganism, by β-phosphogalactosidase (β-Pgal) into glucose and galactose-6-phosphate. Glucose is catabolised by the Embden-Meyerhoff-Parnas (EMP) pathway (commonly known as Glycolysis) **(Boylston, 2012)** and Galactose-6-phosphate is dephosphorylated and then is catabolized via the Tagatose pathway; but some of it will remain unmetabolized and it is excreted from the microbial cell (this system is used by *L. bulgaricus*) **(Daryaei *et al.*, 2006; Ghoddusi, 2011; Tamime and Deeth, 1980; Tamime and Robinson, 1999; Zourari *et al.*, 1992)**.

In the second system, after lactose enters the cell via a permease as an unphosphorylated disaccharide, it is hydrolysed by β-galactosidase (β-gal) to glucose and

galactose. This system is used by both starter cultures **(Daryaei *et al.*, 2006; Zourari *et al.*, 1992)** (see **Figure 2**).

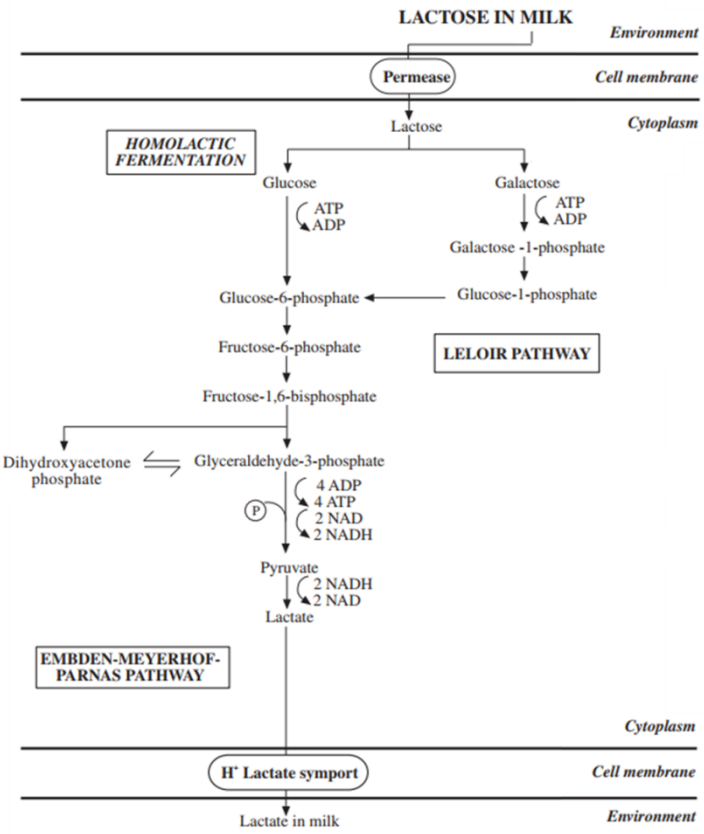


Figure 2 - Homolatic Fermentation of lactose by the yoghurt starter cultures after translocation by permease. Adapted from **Marshall and Tamime, (1997)** and **Tamime and Robinson, (1999)**.

Note that when all glucose is depleted or low concentrations of lactose are present, *S. thermophilus* and *L. bulgaricus* use galactose via the *Leloir* pathway with galactokinase as the first enzyme of the metabolic pathway **(Tamime and Deeth, 1980)**.

In both systems the glucose and galactose converge at dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, where the three-carbon sugars become further oxidised to PEP and then pyruvate kinase produces pyruvate, which is converted into lactic acid by lactate dehydrogenase (LDH) and two types of lactate isomers, L and D. *S. thermophilus* produces mainly L-(+)-lactic acid **(Garvie, 1978; Hemme *et al.*, 1981)** and D-(-)-lactic acid is produced mainly by *L. bulgaricus* **(Tamime and Deeth, 1980)**. L-(+)-lactic acid is usually present in yoghurt at higher amounts than D-(-)-lactic acid **(Chandan and O'Rell, 2013; Tamime and Robinson, 2007)**. The mutual stimulation of the yoghurt cultures through their metabolic activity considerably increases the formation of lactic acid at a rate greater than

would be possible by the individual cultures. *S. thermophilus* and *L. bulgaricus,* in addition to use the lactose in the milk to produce lactic acid, they also synthesize other important flavour compounds, that will be discussed later in **section 1.4.5**. The equation about LAB homofermentative activity from relatively to the formation of lactic acid from lactose is represented in **Equation 1 (Ghoddusi, 2011; Simpson *et al.*, 2012)**.

$$\text{Lactose} + 4\text{ADP} + 4\text{H}_3\text{PO}_4 \rightarrow 4\text{Lactic Acid} + 4\text{ATP} + 3\text{H}_2\text{O} \quad \textbf{Equation 1}$$

The production of acid by LAB has a significant impact on the safety and quality of the cultured dairy products, in other hand, the reduction in pH increases the shelf life and safety of the cultured dairy products through the inhibition of spoilage and pathogenic microorganisms. Acid production by LAB is critical for the precipitation of the casein proteins. These bacteria may also contribute to the degradation of proteins and lipids through proteolytic and lipolytic reactions to further develop the unique texture and flavour characteristics of the cultured dairy products, that will be discussed in **section 1.4.4 (Hill and Kethireddipalli, 2013; Simpson *et al.*, 2012; Tamime and Robinson, 1999)**.

1.4.3. Production of exopolysaccharides

Exopolysaccharides (EPS) materials in yoghurt are constituted by carbohydrates produced by some strains that enhance the body of yoghurt (increasing viscosity and texture) and provide health benefits to consumers, including prebiotic effects, immunostimulatory and anti-tumoral activities, and reduced blood cholesterol levels **(Aryana and Olson, 2017; O'Connor *et al.*, 2006; Tamime and Robinson, 1999; Zhou *et al.*, 2019)**. EPS also can be classified as heteropolyssacarides composed of either linear or branched repeating units varying in size from di- to hepta- saccharides **(Tamime and Robinson, 1999)**.

L. bulgaricus has been shown to produce EPS that contain galactose, glucose, and rhamnose in an approximately 4:1:1 molar ratio and with a molecular weight of approximately 500,000 **(Cerning *et al.*, 1986)**, *S. thermophilus* has been shown to produce EPS consisting primarily of galactose and glucose with smaller amounts of xylose, arabinose, rhamnose, and mannose **(Cerning *et al.*, 1988)**. However, there are different

information about EPS composition produced by these bacteria both in types of sugars and their ratio **(Tamime and Robinson, 1999)**.

EPS formation is influenced by many factors such as the growth medium used, the incubation temperature, the level of acidity in the growth medium and the strain variation **(Cerning, 1995, 1990; Gassem *et al.*, 1997; Grobben *et al.*, 1998)**. Beyond these, there are further factors relating to yield and production of EPS, so it is important a good starter selection and control the cultural conditions **(Vedamuthu, 2006)**. In general, the amount of EPS material produced by the yoghurt microorganisms may reach up to 40 mg/100 mL **(Cerning, 1995)**. Relatively to *S. thermophilus* the optimum yield of EPS production, ≈ 10 mg/ 100 mL, was obtained when the organism was incubated at 30°C for 24 hours and for *L. bulgaricus* when the organism grew at 37°C for 24 h (12 mg/100 mL) **(Mozzi *et al.*, 1995)**. Other difference between these bacteria is the phase of EPS production: in *S. thermophilus*, EPS was reported to be produced in the stationary phase **(Gancel and Novel, 1994)** whereas in *L. bulgaricus* was produced in the exponential phase **(Bouzar *et al.*, 1996)**.

1.4.4. Enzymatic Activity

1.4.4.1. Proteolytic Activity

L. bulgaricus has important protease activity and hydrolyses the milk proteins, caseins, to small peptides and amino acids. These peptides and amino acids enhance the growth of *S. thermophilus*, which has limited proteolytic activity. *S. thermophilus* has peptidase activity and can hydrolyse the intermediate products of casein proteolysis by *L. bulgaricus*. So, during the fermentation, LAB causes a significant degree of proteolysis and this activity may be important for the following reasons: **i)** The liberation of peptides of varying sizes and free amino acids by enzymatic hydrolysis of milk proteins may affect the physical structure of the yoghurt because they may be involved during gel formation; **ii)** Amino acids and peptides may not contribute directly towards the flavour of yoghurt, but they act as precursors for the multitude of reactions which produce flavour compounds **(Tamime and Robinson, 1999)**, such as acetaldehyde (**Boylston, 2012**); **iii)** Bitterness in yoghurt is usually attributed to the production of bitter peptides by the proteolytic activity of *L. bulgaricus*; however, fermentation of the milk at 44°C produces a yoghurt which is less likely to be bitter than yoghurt produced at 38°C **(Aryana and Olson, 2017)**.

Nevertheless, the proteolytic activity of the yoghurt organisms appears to be the most intense activity during the log phase and decreases during cold storage or after the stationary phase has been reached. The ratio of *S. thermophilus* and *L. bulgaricus* in the starter culture (with ratio 1:1 there are more amino acids liberated) and the storage period can also affect the level of amino acids in yoghurt **(Tamime and Deeth, 1980)**. Furthermore, free fatty acids (FFA) can reduce the proteolytic activity of the starter cultures and can affect the texture of the coagulum **(Tamime and Deeth, 1980)**. Due to the proteolytic activity of *L. bulgaricus* and *S. thermophilus*, the profile of nitrogenous compounds in yoghurt is very different when compared with milk, both during fermentation period and during the cold storage of the product.

1.4.4.1. Lipolytic Activity

The triacylglycerol lipase enzymes in yoghurt may originate from starter cultures or from contaminants that survived the heat treatment **(Deeth and Fitz-Gerald, 1976)**. The extent of lipolysis in homogenised milk is higher than in non-homogenised milk due to the destruction of the protective layer of fat globule, where lipases are placed **(Tamime and Robinson, 2007)**. In fact, hydrolysis of fat by the yoghurt starter cultures contribute towards the flavour and Formisano *et al.* (1974) **(***cited by* **Tamime and Deeth, 1980)** reported appreciable loss of lipids, namely a decrease of 3.4 % (determined gravimetrically) or 6.6 % (determined colorimetrically) in the fat in yoghurt stored for 21 days at 4°C and also noted an increase of free fatty acids, approximately 3.738 %.

Fermentation of full fat milk with *S. thermophilus*, *L. bulgaricus* or *L. acidophilus* resulted in different effects on milk lipids, and, according to **Rao and Reddy, (1984)**, there is a significant increase in saturated fatty acids (SFA) and oleic acid (C18:1 c9) and a decrease in linoleic (C18:2 c9, c12) and linolenic acids (C18:3 c9, c12, c15) in the glyceride fraction. Thus, the increase of FFA was moderate, but in other hand the monoglyceride fraction disappeared completely upon fermentation and the changes in cholesterol content are not significant **(Tamime and Robinson, 1999)**.

During the manufacture and storage of yoghurt, there is an appreciable increase in the total level of volatile fatty acids (VFA) in the product and *L. bulgaricus* produces more VFA than *S. thermophilus*. This increase depends on several variables, such as the strains of the starter bacteria, type of milk, duration and temperature of incubation, temperature of heat

treatment of the milk and/or the age of yoghurt **(Dutta *et al.*, 1973, 1971; Kuila *et al.*, 1971; Singh *et al.*, 1980)**. However, if milk has low concentration of citric acids, is observed a slight decrease in VFA **(Dutta *et al.*, 1972)**. Despite this, the higher production of VFA by *L. bulgaricus* is probably due to endopeptidases and/or exopeptidases activity rather than lipases **(Tamime and Robinson, 1999)**. **Table 5** shows the changes in VFA when milk is fermented at 37°C for 24 h and 72 h, with these two bacteria.

Table 5 - Changes in volatile fatty acids (VFA) in whole (W) and skimmed (S) milk fermented at 37°C for different durations with yoghurt organisms (24 and 72 h). Adapted from **Tamime and Deeth, (1980)** and **Tamime and Robinson, (1999)**.

Fatty Acids		**Milk**	***L. bulgaricus* + *S. thermophilus***	
			24 h	**72 h**
Total VFA (mg/100g)	W	3.20	6.05	6.26
	S	2.97	5.89	6.32
Acetic acid (C2)	W	0.21	0.57	0.48
	S	0.20	0.12	0.20
Propionic acid (C3)	W	-	0.22	0.11
	S	-	-	-
Isobutyric acid (i-C4)	W	0.03	0.13	0.14
	S	0.03	0.03	0.06
n-butyric acid (n-C4)	W	0.39	1.05	1.44
	S	0.38	0.66	1.08
Isovaleric acid (i-C5)	W	0.05	0.15	0.06
	S	0.03	0.07	0.17
n-hexanoic acid (n-C6)	W	1.09	1.56	2.57
	S	1.13	2.40	2.04
Octanoic acid (C8)	W	0.97	1.78	1.64
	S	0.96	2.26	2.36
Decanoic acid (C10)	W	1.21	2.65	2.22
	S	1.10	3.11	2.92

1.4.4.2. Urease Activity

During milk fermentation, LAB are faced with constantly changing environmental stimuli and stresses, which can affect their cellular physiology. These predictable environmental changes include pH variations, the limitation of nutrient availability, and the accumulation of toxic metabolites (i.e., lactic acid) formed by the fermentation process. Exposure to low pH for a long period of time causes an arrest of growth, a dramatic reduction

of glycolytic fluxes and a progressive loss of viability **(Cotter and Hill, 2003; Hutkins and Nannen, 1993; Siegumfeldt *et al.*, 2000)**.

Urease react as a stress response that is activated to counteract acidic environmental pH in *S. thermophilus* and the urease content increases as consequence of increase glycolytic flux **(Arioli *et al.*, 2010)**. Outside of the "selfish" utility of urease for cells, there is a cooperative relevance of urease in an ecological context: provides a local benefit because other individuals can take advantage of the release of ammonia (NH_3) and carbon dioxide (CO_2) from urea, namely *L. bulgaricus* **(Arioli *et al.*, 2010; Monnet *et al.*, 2005; Tinson *et al.*, 1982)**. As stated in **section 1.4.1** there is a cooperation between these two bacteria that is illustrated into **Figure 3**, where CO_2 and usually formic acid (and other acids) are released by the breakdown of urea in the milk by urease, thus stimulating the growth of *L. bulgaricus*.

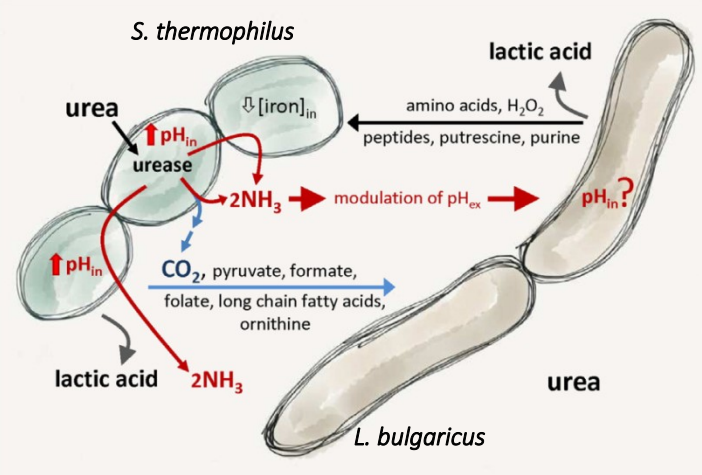


Figure 3 - Schematic representation of the molecular interactions that play key roles in the mutualistic behaviour of the yoghurt consortium. The effect of *S. thermophilus* urease in urea hydrolysis is shown in blue and hypothetical role of the NH_3 released by this enzyme in the pH_{in} of *L. bulgaricus* are shown in red. Adapted from **Arioli *et al.* (2010).**

1.4.5. Production of flavour compounds

Yoghurt's popularity as food largely depends on its sensory characteristics, with aroma and taste being the most important. Yoghurt is well liked for its delicate and low intense acidic flavour **(Ott *et al.*, 1997)**. So, flavour is an important factor determining food product acceptability and preference for consumers **(Cheng, 2010)**. Starter cultures are primarily responsible for producing the flavour compounds, which contribute to the aroma of yoghurt. These compounds may be divided into four main categories: Non-volatile acids (e.g. lactic,

pyruvic, oxalic, and succinic); Volatile acids (e.g. acetic, propionic and butyric); Carbonyl compounds (e.g. acetaldehyde, acetone, acetoin and diacetyl); Miscellaneous compounds (e.g. certain amino acids and compounds derived from protein, fat and lactose degradation) **(Tamime and Robinson, 1999)**.

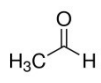
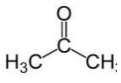
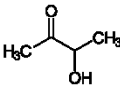
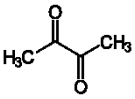
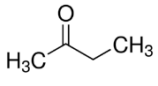
More than ninety flavour volatile compounds have been identified in yoghurt including carbohydrates, alcohols, aldehydes, ketones, acids, esters, lactones, sulfur compounds, pyrazines, and furan derivatives **(Cheng, 2010; Ott *et al.*, 1997)**, as displayed in **Table 6** and **Table 7**. **Dan *et al.*, (2017)** used solid-phase microextraction (SPME) (more sensitive than other conventional methods) to extract compounds and gas chromatography-mass spectrometry (GC-MS) methods to identify volatile compounds produced by *S. thermophilus* and *L. bulgaricus* individually, and both species together in yoghurt. Acetaldehyde, ethanol, acetone, diacetyl and 2-butanone have high impact on the desired production flavour and are present in detectable amounts **(Ulberth, 1991)**. Each of them has a characteristic odour that contributes for the final flavour of the product. All of these, except ethanol, are reported responsible for imparting desirable flavour to yoghurt are the carbonyl compounds – which are present in relatively high concentrations (in decreasing order –**Table 7**) (**Imhof *et al.*, 1994; Kaminarides *et al.*, 2007**).

Acetaldehyde is a key flavour component of yoghurt described as having a fruity aroma as be descripted in **Table 7 (Hill and Kethireddipalli, 2013; Simpson *et al.*, 2012)**. Production of acetaldehyde takes place via several pathways, which use different compounds as precursors, such as glucose, catechol, glyceraldehydes, acetylene, threonine, glycine, and even deoxyribonucleic acid (DNA) (**Chaves *et al.*, 2002; Tamime and Robinson, 2007; Zourari *et al.*, 1991**). Breakdown of threonine to acetaldehyde and glycine is reported as the major pathway and the reaction is catalysed by the enzyme threonine aldolase, present in both *L. bulgaricus* and *S. thermophilus*.

Table 6 - List of volatile compounds that have been identified in plain yoghurt. Adapted from **Cheng (2010).**

Carbonyl compounds	*Alcohols*	*Sulfur compounds*	*Heterocyclic compounds*
Acetaldehyde	Methanol	Dimethyl sulfide	Furan
Acetone	Ethanol	Dimethyl disulfide	Furfural
Propanal	1-Propanol	Dimethyl trisulfide	2-Methylfuran
2-Propanone	2-Propanol	S-methyl thioacetate	2-Pentylfuran
Butanal	1-Butanol	Methional	2-Furanmethanol
2-Butanone	2-Butanol	Tetramethyl thiourea	Pyrazine
Diacetyl	2-Methyl-1-propanol	*Nitrogen compounds*	Methylpyrazine
Acetoin	Cyclobutanol	N,N-dimethylformamide	Pyrrole
Pentanal	1-Pentanol	Lactamide	1-Methylpyrrole
2-Methylbutanal	3-Pentanol	N-ethyl-benzenamine	2-Methyl tetrahydrofuran-3-one
3-Methylbutanal	1-Penten-3-ol	*Hydrocarbons*	2-Methylthiophene
3-Methyl-2-butenal	3-Methyl-2-butenol	Heptane	2-Methyltetrahydrothiophen-3-one
2-Pentanone	3-Methylbutanol	Methylcyclohexane	Benzothiazole
3-Penten-2-one	Pentan-2-one-4-ol	Nonane	Methyl 2-piperidine
2-Hydroxy-3-pentanone	3-Methyl 3-cyclohexenol	Undecane	Furfuralcohol
2,3-Pentanedione	2-Ethyl hexanol	*Aromatic compounds*	1,2-Dihydro-2,2,4-Trimethylquinoline
Hexanal	2-Buthyl octanol	Benzene	2,3-dihidro-1,3,3-trimethyl 1H-Indole
2-Hexanone	Guaiacol	Toluene	*Terpene*
3-Hexanone	*Acids*	Ethylbenzene	L-limonene
Heptanal	Acetic acid	1,3-Dimethylbenzene	*Others-solvent contamination?*
2-Heptanone	Propionic acid	1,4-Dimethylbenzene	Acetonitrile
3-Heptanone	Butyric acid	1,2-Dimethylbenzene	Dichlorometane
Octanal	2-Methylpropanoic acid	Ethenylbenzene	Trichloromethane
3-Octanone	Pentanoic acid	Propylbenzene	
1-Octen-3-one	Isovaleric acid	Trimethylbenzene	
1-Nonen-3-one	Hexanoic acid	1-Methyl ethenylbenzene	
Nonanal	Heptanoic acid	1-Ethyl-4-methylbenzene	
(E)-2-Nonenal	Octanoic acid		
2-Nonanone	Nonanoic acid		
Decanal	Decanoic acid		
Undecanal	Benzoic acid		
2-Undecanone	*Esters*		
2-Dodecanone	Methyl formate		
2-Pentadecanone	Methyl acetate		
γ-Dodecalactone	Ethyl acetate		
δ-Dodecalactone	Butyl acetate		
Benzaldehyde	Diehyl phthalate		
Phenylacetaldehyde			

Table 7 - Production of carbonyl compounds (% g/g) by yoghurt starter cultures and their odour descriptor. Adapted from **Tamime and Robinson (1999)** and **Cheng (2010).**

Volatile Compounds		**Acetaldehyde** 	**Acetone** 	**Acetoin** 	**Diacetyl** 	**2-butanone** 
Produced by	*S. thermophilus*	1.0 - 13.5	0.2 - 5.2	1.5 - 7.0	0.1 - 13.0	*
	L. bulgaricus	1.4 - 77.5	0.3 - 3.2	Trace - 2.0	0.5 - 13.0	*
Odor descritor		Ethereal, fresh, green, pungent	Sweet, fruity	Buttery	Buttery, creamy, vanilla	Varnish-like, sweet, fruity

(*) Not described;

At the temperatures 40–45°C used in yoghurt manufacture, *L. bulgaricus* is the main contributor of threonine aldolase (**Zourari *et al.*, 1991**) because this enzyme is inactivated in *S. thermophilus* at 30–42°C **(Wilkins *et al.*, 1986)**. Moreover, threonine aldolase activity is also influenced by glycine level, salts and some divalent cations **(de Nadra, 1987; Marranzini *et al.*, 1989; Schmidt *et al.*, 1989, 1983; Wilkins *et al.*, 1986)**. Furthermore, *S. thermophilus* and *L. bulgaricus* have no alcohol dehydrogenase, and because of that acetaldehyde is not reduced to ethanol and it is accumulated **(Simpson *et al.*, 2012)**. The production of acetaldehyde, during the manufacture of yoghurt starts only when pH reaches to 5.0, but the maximum production is at pH 4.2 and stabilises at pH 4.0. Moreover, the fortification of milk base with milk solids can significantly increase the acetaldehyde content of the yoghurt **(Gorner *et al.*, 1968)**. The content of acetaldehyde in yoghurt and its losses after storage for 24 h also depends of milk origin, i.e. different mammalian milk produces different content of acetaldehyde **(Yaygin and Mehanna, 1988; Yu and Nakanishi, 1975)**.

Relatively to diacetyl, it is derived from both lactose and citrate. Acetoin is readily produced from diacetyl by the enzyme diacetyl reductase **(Cheng, 2010)** (see **Figure 4**). Small quantities of acetone and 2-butanone are usually originate from milk, but certain quantities are produced by the yoghurt bacteria **(Andreas Ott *et al.*, 1999; Gallardo-Escamilla *et al.*, 2005; Georgala *et al.*, 1995)**.

All manufacturing factors such as the source of milk (e.g. cow, sheep, or goat), processing techniques, added components (stabilizers, fruits, flavourings, probiotics, and prebiotics), packaging materials, and storage conditions have an impact on the final taste and aroma of yoghurt **(Routray and Mishra, 2011)**. Further, undesired odorants can be produced during the storage of yoghurt, and as in any other fat-rich dairy product, being lipid oxidation the major contributor to these off-flavours.

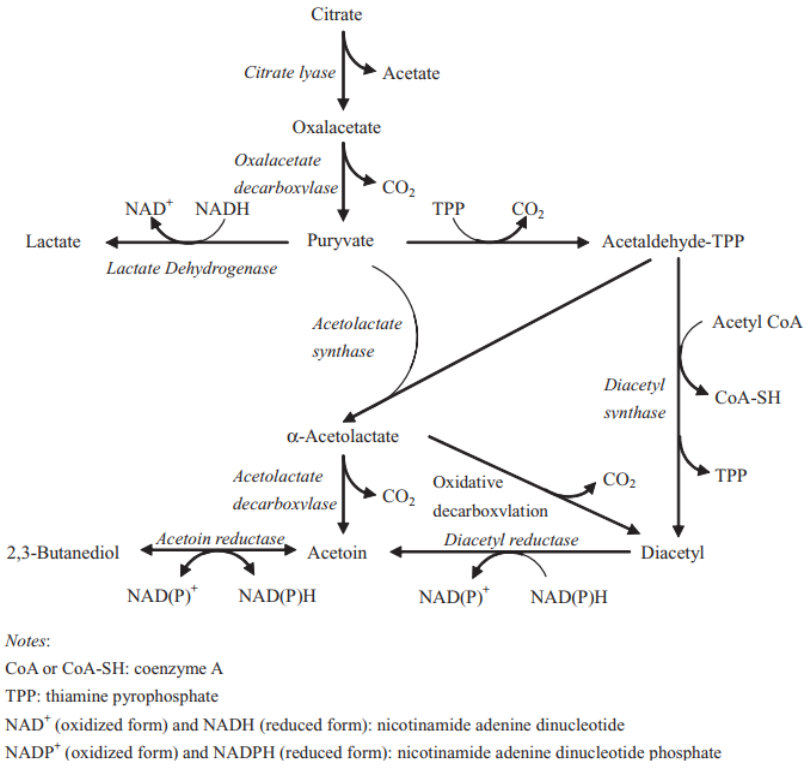


Figure 4 - General metabolic pathways of citrate during milk fermentation to produce some volatile compounds. Adapted from **Cheng (2010)**.

1.4.6. Production of antimicrobial compounds

LABs have antimicrobial systems that offer for the development of an effective natural preservation process. The low molecular weight compounds elaborated by these bacteria are called bacteriocins and have an inhibitory effect over spoilage organisms in yoghurt, cheese and other fermented foods. In addition of inhibition of growth of spoilage organisms, that result in an increase of shelf life of product, these compounds can also add health benefits to fermented foods. The bacteriocins produced by LAB in ribosomes are considered as GRAS and have a potential application as consumer friendly bio preservatives **(Ghanbari and Jami, 2013; Khurana and Kanawjia, 2007)**.

Research on bacteriocins from LAB has expanded during last decades and is known that bulgarican bacteriocin is synthesized by *L. bulgaricus* and *S. thermophilus* can synthesize thermophilin 13, a peptide with molecular weight (MW) of approximately 4,000 g/mol, that is thermostable and active at different pH values **(Marciset *et al.*, 1997)**.

1.5. Yoghurt Composition

As defined by the FDA, a yoghurt or a food that contains 10 % of the required daily intake (RDI) of a specific nutrient (e.g., calcium) is considered a good source of that nutrient, while if it contains 20 % or more of the RDI, it is considered an excellent source. There are requirements regarding the composition of the product with respect to fat content, acidity, and amounts of non-fat milk solids, mainly protein content. Relatively to the nutrient composition yoghurt and milk are very similar, however, yoghurt is more concentrated in riboflavin, vitamin B12, calcium, magnesium, potassium and another nutrients content. Low-fat yoghurt contains approximately 25 % more potassium, calcium and magnesium compared with an equal serving of low-fat milk due to their release into the caseins during processing. However the nutrient profile of yoghurt depends of the original nutrition profile of milk and also the fermentation process, furthermore other nutrients can be added **(Chandan, 2017; Freitas, 2017; Hill and Kethireddipalli, 2013)**. Yoghurt can also be an excellent source of high-quality protein, but the nutritional value of proteins differs substantially depending on their essential amino-acid composition and digestibility. In **Table 8** is represented a summary of nutrient content of plain low-fat yoghurt compared with whole milk.

Table 8 - Nutrient content of 100 g of yoghurt products compared with milk. Adapted from **Chandan, (2017)** and **Bottazzi *et al.* (1998).**

Nutrients	Unit	Whole milk 8 g Protein/8 oz	Plain yoghurt low fat 12 g Protein/8 oz
Moisture	%	87.90	85.07
Energy	Kcal	61	63
Protein	g	3.47	5.25
Fat	g	3.25	1.55
SFA	g	2.096	1.00
MUFA	g	0.893	0.426
PUFA	g	0.092	0.044
Cholesterol	mg	13	6
Carbohydrate	g	4.66	7.04
Dietary fiber	g	0	0
Calcium	mg	121	183
Iron	mg	0.05	0.08
Magnesium	mg	12	17
Phosphorus	mg	95	144

Potassium	mg	155	234
Sodium	mg	46	70
Zinc	mg	0.59	0.89
Vitamin C	mg	0.5	0.8
Thiamine (B1)	mg	0.029	0.044
Riboflavin (B2)	mg	0.142	0.214
Niacin (B3)	mg	0.075	0.114
Pyridoxine (B6)	mg	0.032	0.049
Folic Acid (B9)	mg	7	11
Cobalamin (B12)	mg	0.37	0.56
Pantothenic acid (B5)*	µg/100g	482	381
Vitamin A	IU	99	51
Vitamin D	IU	2	1
Vitamin E	mg	0.06	0.03
Vitamin K	µg	0.2	0.2

Saturated fatty acid (SFA); Monounsaturated fatty acid (MUFA); Polyunsaturated fatty acid (PUFA); 1 oz = 28.3495231 g;

1.6. Yoghurt Health Benefits

Consumption of dairy products plays a role in building a nutrient-dense diet and contributes to a healthy lifestyle. Several studies have been done regarding the relationship of yoghurt with human health and some of the conclusions include **(Tamime and Robinson, 1999)**: **i)** Increase calcium and protein intake - which promotes satiety, helps in maintaining a healthy body weight, and helps muscle and bone growth; **ii)** Increase other nutrients and vitamins intake; **iii)** Help in digestive tract at digestibility of lactose – 70 % of the world's population exhibit various degrees of lactose malabsorption; **iv)** Decrease faecal enzyme activity and the survival yoghurt organisms in human stomach adheres to intestinal tract; **v)** Prevention/treatment of: acute diarrhoea; rotavirus diarrhoea; antibiotic-induced diarrhoea; v**i)** Yoghurt consumers presented a lower prevalence of hypertriglyceridemia and low high density lipoprotein (HDL) cholesterol plasma levels, which are metabolic syndrome components **(Mena-Sánchez *et al.*, 2018)**.

1.7. Yoghurt storage

Various microbial, enzymatic, or chemical reactions occurring within yoghurt during storage may alter its physical, chemical, and microbiological characteristics, causing deterioration or spoilage **(Cheng, 2010)**. Yoghurt and probiotic fermented milk are beneficial to human health because of the type of bacteria and the large number of viable cells they should contain. Although quantitative standards vary from 10^6 to 10^7 CFU/g viable cells as minimum requirements, it is generally recommended that yoghurt or fermented milk should contain at least one million viable cells per gram at the time of consumption, for that it is important to test bacteria for growth and viability during cold storage to find out if these numbers are maintained **(Oliveira *et al.*, 2006)**. For that propose, **Damin *et al.*, (2008)** studied the average initial microbial counts and during storage at 10°C in a skimmed yoghurt. It was noted that *S. thermophilus* maintain its concentration for 35 days as opposed to *L. bulgaricus*, that visibilly descrease (**Figure 5**).

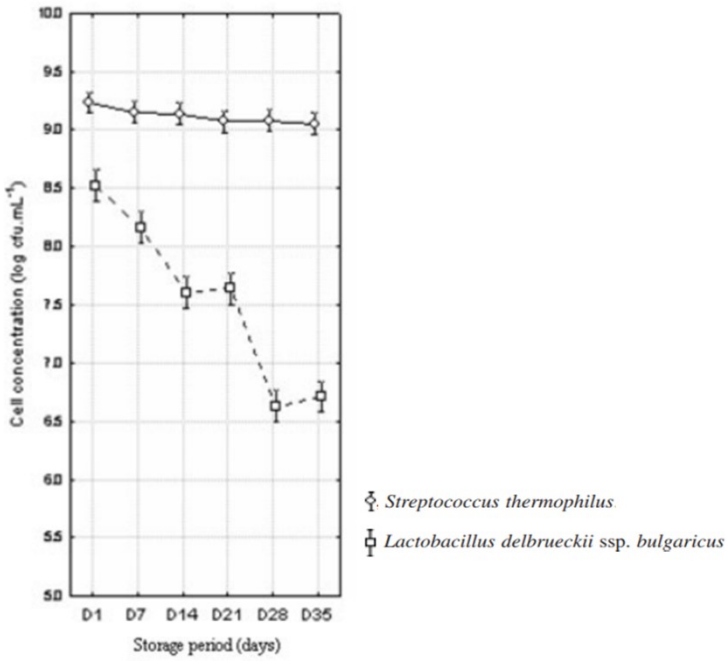


Figure 5 - Cell concentration in fermented milk samples after 1 day (D1) of fermentation, and during cold storage of the products for 35 days. Adapted from **Damin *et al.* (2008)**.

Furthermore, several factors affect bacterial viability, such as post fermentation acidification, acidity and dissolved oxygen (**Table 9**). Due to the high acidifying rate of these bacteria, higher acid content was produced during storage (by 35 days) **(Damin *et al.*, 2008)**.

Table 9 - pH, titratable acidity and dissolved oxygen variations determined during post fermentation in yoghurt. Adapted from **Damin *et al.* (2008)**.

Storage period (days)	pH post fermentation	Titratable acidity (TA) (% lactic acid)	Dissolved oxygen (ppm)
Milk	-	-	≈ 4.0
1	4.35	1.27	≈ 1.4
7	4.19	1.33	≈ 8.0
14	4.18	1.38	≈ 10.0
21	4.03	1.42	≈ 9.5
28	4.04	1.41	≈ 9.0
35	4.05	1.34	≈ 8.0

With the same objective, **Salvador and Fiszman, (2004)** studied alterations during long storage (10°C, 20°C and 30°C for 91, 21 and 3 days, respectively) of whole and skimmed flavoured set-type yoghurt, and, in the case of storage at 10°C, LAB did not decrease as in the yoghurt studied by **Damin *et al.*, (2008)**, as can see in **Table 10**, and pH values ranging from 4.21 to 3.96 and from 4.27 to 4.01 for whole and skimmed yoghurt, respectively were observed. **Salvador and Fiszman, (2004)** also studied yoghurt syneresis during 3 different storage temperatures and this increase was more noticeable during the first days of storage and slowly increasing up to 40 days and keeps its contents in serum from this day. These authors also observed low syneresis when yoghurt was stored at 10°C. Furthermore, whole yoghurt (less than 4 mL/125 g) showed more syneresis than skimmed yoghurt (less than 2 mL/125 g) after 10 days of storage.

Table 10 - Viability (in log CFU/mL) of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* of whole and skimmed yoghurt during storage at 10°C. Adapted from **Salvador and Fiszman (2004).**

Time (days)	Whole yoghurt		Skimmed yoghurt	
	S. thermophilus	*L. bulgaricus*	*S. thermophilus*	*L. bulgaricus*
0	8.6	8.6	8.9	9.0
15	8.6	7.8	8.7	8.7
35	8.5	8.1	8.6	6.6
49	8.3	7.1	7.8	7.4
63	6.7	4.7	7.0	6.8
77	5.2	3.5	6.2	5.4
91	4.2	4.2	5.9	5.2

Rheological properties of yoghurt during cold storage is also an important parameter to be evaluated, as these parameters provide information on properties that may affect their consistency during consumption and their resistance to processing, such as stirring or pumping in the production of the stirred yoghurt **(Damin *et al.*, 2008)**. Viscosity is correlated with the structural state of the material, and **Damin *et al.*, (2008)** determined several rheological parameters and observed small difference during cold storage, namely an increase viscosity at 35th day. Furthermore, the same authors characterized consistency at rest as a function of stability during storage. G' (elastic modulus) values were higher than G'' (viscous modulus), indicating an elastic characteristic that gives better stability during storage **(Damin *et al.*, 2008)**, and this could be due to ongoing fusion of casein particles **(Lucey and Singh, 1997)**. Firmness during cold storage ranged from 0.70 to 0.92 Newton (N) and **Oliveira *et al.*, (2006)** verified that longer fermentation times resulted in greater firmness in case of yoghurt produced with skimmed milk powder (**Table 11**). **Salvador and Fiszman, (2004)** also determined yoghurt firmness and concluded that skimmed yoghurt is firmer (less than 0.6 N) and more adhesively than whole yoghurt (less than 0.4 N).

Table 11 - Rheological parameters analysed in skimmed yoghurt fermented with *L. bulgaricus* and S. thermoplilus. Adapted from **Damin *et al.* (2008)**.

Storage period (days)	LVE (Pa)	G' (Pa)	G'' (Pa)	Structural recuperation (%)	Firmness (N)
1	1.68 x 10	2.60×10^2	7.10×10^1	20.70	0.92
7	1.19 x 10	4.30×10^2	1.10×10^2	21.10	0.69
14	-	-	-	-	0.50
21	2.42 x 10	5.20×10^2	1.20×10^2	19.90	0.68
28	-	-	-	-	0.73
35	9.77×10^{-1}	5.60×10^2	1.30×10^2	21.20	0.70

Linear viscoelastic (LVE); Elastic modulus (G'); Viscous modulus (G''); Structural recuperation (% of G' recuperation calculated by thixotropy analysis);

Generation of volatile by-products leads to off-flavours and makes the product unsatisfactory for consumers, and the evolution of volatile compounds can often determine the shelf life of yoghurt **(Cheng, 2010)**. For this reason, a quantitative study of aroma compounds may also help in the creation more suitable and stable flavours for yoghurt. Routine analysis of the key aroma compounds can be used for quality monitoring during yoghurt production and the analysing of the profile of volatile compounds in yoghurt can be used as a parameter to provide consumers with better quality and safer food **(Cheng, 2010)**. For that propose, **Dan *et al.*, (2017)** studied the variation of volatile compounds during storage at 4°C for 14 days in yoghurt produced with sterile milk prepared by reconstitution of 10 % (weight/volume) (w/v) skimmed milk powder in distilled water fermented with *S. thermophilus* IMAU80842 and *L. bulgaricus* IMAU20401, and concluded that:

- ✓ **Volatile compounds:** acetic acid, hexanoic acid, cyclohexanecarboxylic acid and octanoic acid (increase during 14 days of storage); acetic anhydride, butanoic acid and 3-methylbutanoic acid (decreased during 14 days of storage);
- ✓ **Aldehyde compounds:** acetaldheyde decreased its content during storage;
- ✓ **Ketone compounds:** all decreased their content during storage, namely: 2-pentanone, 2-heptanone; 2-nonanone; 2-undecanone;
- ✓ **Alcohol compounds:** 1-haxanol and 2-nonanol increase their content and 1-heptanol decrease. Other alcohols were found to be present in a single day of storage;

- ✓ **Ester compounds:** just acetic acid ethenyl ester have been every identified during the 14 days of storage and their content decreased;
- ✓ **Hydrocarbon compounds:** just *o*-xylene appears until the 3rd day of storage to increase but isn't detect at 7th day. Other compounds were identified, only once in a single day;

Beyond these alterations during yoghurt storage, other parameters as final pH, incubation temperature and LAB strains can affect the final product and cause changes during storage. **Beal *et al.*, (1999)** studied a great number of combinations to conclude about the changes of yoghurt during storage. For that, the authors produced yoghurt with skim milk (fortified with skim milk powder) and pasteurized it at 80°C for 30 minutes. Then, inoculated 50 % (volume/volume) (v/v) of *L. bulgaricus* and 50 % (v/v) of *S. thermophilus*, but the last one LAB can have ropy (R) proprieties or acidifying (A) proprieties and be added in different ratios: A/R = 25/25 or 0/50 or 50/0. Also, was used different incubation temperatures (39, 42 and 45°C) and reached two pH final (4.4 and 4.8). This combinations produce different yoghurts and they was storage at 4°C for 21 days and analysed at 1st, 7th and 21st storage day:

- ✓ During storage, pH decreases more when the final pH was 4.8 than 4.4 and the main decrease in pH occurred between the 0 and 7th days because of the lactose consumption and lactic acid and galactose production. Bacterial concentrations fell by 40 to 75 %, especially between 7th and 21st day of post-acidification. This decrease is more pronounced in case of *S. thermophilus*.
- ✓ Relatively to the bacterial concentrations, were influenced by storage time, final fermentation pH incubation temperature and storage time. In all cases, *S. thermophilus* content was higher than *L. bulgaricus* content. The final concentration of *S. thermophilus* depended on the strain used with *S. thermophilus* A showing higher grow than *S. thermophilus* R. Incubation temperature did not affect *L. bulgaricus* growth, but in the case of *S. thermophilus*, lower temperatures as 39 and 42°C improved their growth but is strain dependent. The higher the temperature of fermentation was, the lower the time necessary to reach the maximal acidification value was. The final pH significantly influenced bacterial concentrations: concentrations of *L. bulgaricus* were higher in yoghurts that stopped at pH 4.4 and

for *S. thermophilus* is at pH 4.8, because *S. thermophilus* is more sensitive to acidic pH.

- ✓ Relatively to the texture: strain association, temperature and final pH has significant effects on yoghurt viscosity. Higher viscosity was obtained with ropy cultures (R) because their higher EPS production and, texturing character increasing with decreasing temperature and final pH. Yoghurt texture was not influenced by temperature for *S. thermophilus* A. Viscosity was more influenced by temperature at pH 4.4 (higher firmness) than 4.8. Different fermentation times may affect product viscosity. Between 1st and 7th day of storage there was an increase of viscosity, but between 7 and 21 days, no significant enhancement of texture was observed.

2. Fermentation at non-conventional conditions

The interest in increase yield and productivity in microbial fermentations led to study fermentations at non-conventional conditions. Several strategies have been used to try manipulating metabolic pathways of microorganisms such as high pressure (HP), pulsed electric fields (PEF), ohmic heating (OH) and ultrasounds (US).

Technically, these technologies, when applied, usually lead to microbial inactivation, but in specific stress conditions (milder conditions than those used for microbial inactivation, known as sub-lethal conditions) may activate specific stress response mechanisms that promote their adaptation at new conditions. Some microorganisms were tested under non-conventional conditions, including both bacteria and yeasts, and US was the most studied technology for this purpose. The follow section describes only the HP technology, in what concerns microorganisms that are involved in milk fermentation **(Mota *et al.*, 2018)**.

2.1. High Pressure (HP)

HP is an emerging technology with increasing successful industrial applications as a non-thermal food pasteurization method, since it allows the extension of food shelf life, usually without substantial modification of its nutritional, functional and organoleptic properties **(Barba *et al.*, 2015, 2012)**. Some authors have been studied yoghurt fermentation under pressure and their conclusions are summarized in **Table 12**.

Equipment used for fermentation under high pressure (**Figure 6**) includes a fermenter in a pressure vessel (thick-wall cylinder), in some cases with agitation and temperature control **(Mota *et al.*, 2018)**. The desired pressure is achieved through compression of a pressure-transmitting fluid using the combined action of a pump and an intensifier. The most commonly used pressure-transmitting fluid is water, but glycol, and mixtures of glycol and water, silicone oil, sodium benzoate solution or castor oil may also be used **(Balasubramaniam *et al.*, 2015)**. HP systems have been intensively studied and engineered in the last decades, but some challenges remain, as the need to improve the strength of HP vessels and the capacity of the pumps, as well as the vessel's resistance to many cycles.

Table 12 - Studies about yoghurt fermentation under pressure.

Temperature (ºC)	Pressure (MPa)	Main Results	Reference
43	0.1, 5, 15, 30, 50, 100	Pressure influences negatively the fermentation rate: with the increase of pressure there is a gradual inhibition of fermentation until stops at pressures about 100 MPa. Fermentation under 5 MPa is obtained as final product a yoghurt but fermentation time was twice of process at atmospheric pressure. With the increase of pressure there are an increase of D-glucose in extracellular medium. L-lactic acid was more abundant in yoghurt than D-lactic acid and in the end of fermentation the proportion of both isomers was similar for fermentation at 0.1 and 5 MPa. Acetaldehyde production was inhibited with increasing pressure but not entirely, since at 100 MPa there was still acetaldehyde production. *S. thermophilus* was present in a higher amount than *L. bulgaricus* and it was more resistant to pressure, thus it was concluded that *S. thermophilus* had a more active role in fermentation under pressure.	Lopes, 2013
43	0.1, 5, 15, 30, 50, 75, 100	Fermentation at 5 MPa do not compromises the viability of the bacterial strains (*S. thermophilus*, *L. bulgaricus* and *B. lactis*) but the fermentation time is higher. No fermentation was found in samples subjected to 100 MPa for 180 min but these samples revealed normal metabolic activity when they were returned to atmospheric pressure. Under pressure, the *S. thermophilus* load was always lower than that in the control samples. The counts for the probiotic strain *B. lactis* seem to be nearly constant over fermentation time at 0.1 MPa. However, after 600 min at 5 MPa, the B. lactis load was slightly lower than at 0.1 MPa. *B. lactis* load increased during the first 180 min at 100 MPa, but the load was decreased considerably after that time. However, after 600 min at 100 MPa, there were still viable bacteria.	Mota *et al.*, 2015
35, 43, 50	0.1, 10, 30	*S. thermophilus* was more sensitive to the combination of high temperatures and pressures than *L. bulgaricus*. Both syneresis and texture were influenced by fermentation conditions. Yoghurts fermented at 10 MPa presented syneresis similar to control yoghurts and a firm texture. Yoghurts fermented under 30 MPa had higher syneresis and the yoghurt was less firm.	Lopes *et al.*, 2019a

25, 35, 43, 50	0.1, 10, 30, 50, 100	At 50 and 100 MPa it was not possible to ferment yoghurt at any temperature. At 50°C the fermentation rate was similar for the yoghurts fermented under 0.1, 10 and 30 MPa. Fermentation is accelerated by the temperature increase (until 43ºC) but slowed down by the pressure increase. Fermentation is deaccelerated at 50ºC, but pressure did not slow down fermentation. **Lactose hydrolysis and lactic acid production were not affected by pressure to the same extent:** ✓ At 25ºC, fermentation at 10 MPa presented a higher final lactose concentration than fermentation at 0.1 and 30 MPa, which had similar final values. ✓ At 35°C, the variation of lactose concentration at 30 MPa was slower than at the other pressures tested, while galactose variation was similar. ✓ At 43°C, lactose was more consumed at 0.1 MPa but the final concentrations of galactose were similar for all pressures tested. ✓ At 50°C, lactose was lower consumed at 0.1 MPa, but a lower increase of galactose concentration was observed at 30 MPa. The conditions 10 MPa/25ºC, 10 MPa/43°C, 30 MPa/43°C and 0.1 MPa/50°C presented lower sugars consumption, but at 10 MPa/50°C it was verified a higher consumes. Lactic acid production varies accordingly to the pH variation, but its production was inhibited by increasing pressure at all temperatures tested. Yoghurts fermented under 0.1 and 10 MPa had similar lactic acid content. 10 MPa/43ºC are the best fermentation conditions.	Lopes *et al.*, 2019b

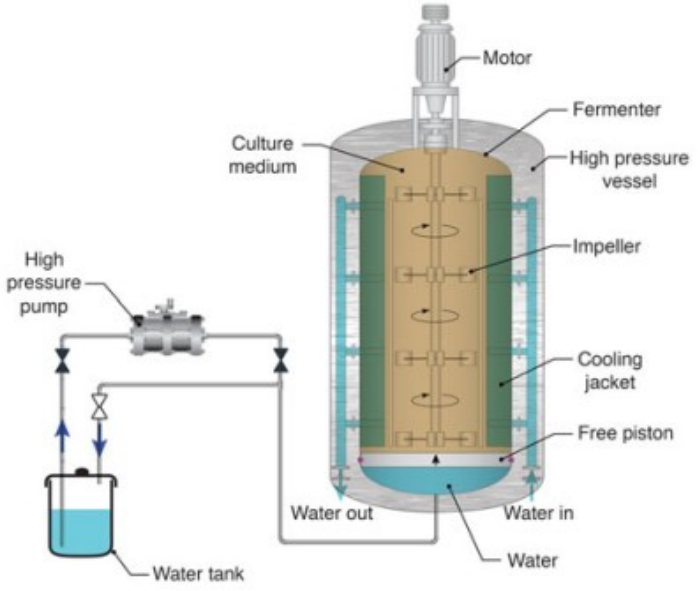


Figure 6 - Schematic representation of microbial fermentation under high pressure used at laboratory scale Adapted from **Mota *et al.* (2018).**

2.1.1. Effect of high pressure on microorganisms

The mechanisms behind microbial inactivation by HP are already well understood, with identification of several effects on cell metabolism, physiology and structural organization **(Bartlett, 2002)**. With increasing pressure, relevant cell structures and functions are successively compromised until it becomes impossible to withstand the stress and survive the hostile conditions **(Mota *et al.*, 2013)**.

In terms of cell structure, different organelles exhibit different sensitivities to HP. For instance, lipid membranes are particularly pressure sensitive because of their high compressible potential. Thus, changes in membrane composition and fluidity are observed under HP, as well as the weakening of important protein–lipid interactions **(Winter and Jeworrek, 2009)**. HP treatments may also affect the structure of DNA, ribosomes and proteins **(Abe, 2007; Macgregor, 2002; Niven *et al.*, 1999)**, possibly leading to inhibition of cell processes (such as replication, transcription and translation) and metabolic reactions essential for cell maintenance. The magnitude of cell damage by HP is highly dependent on several parameters, which include the level and duration of the pressure treatments, the compression method and other environmental conditions (temperature, media composition, pH, etc.). In addition, each microbial strain has a specific degree of HP tolerance according to their intrinsic cellular characteristics. In general, prokaryotes are more HP-resistant than

eukaryotes, gram-positive bacteria are more HP resistant than gram-negative bacteria, and cocci are more HP-resistant than bacilli **(Huang *et al.*, 2014)**.

The cell growth stage was also found to affect microbial tolerance to HP treatments, which is usually higher during the stationary phase than during the exponential phase. This can be explained by the lower stress tolerance of cells during the exponential phase, due to the continuous cell division and synthesis. In contrast, microorganisms in the stationary phase have complete cell structures, thus they can resist more severe stress levels **(Huang *et al.*, 2014; Patterson, 2005)**. Moreover, **Hill *et al.*, (2002)** reported that HP resistance observed during the stationary-phase is partially due to the synthesis of proteins that protect against a range of adverse conditions. It is important to note that microorganisms are more likely to be stressed or injured than killed under HP, particularly when lower intensity treatments are applied **(Huang *et al.*, 2014)**. Several studies have found that microorganisms possess regulatory genes for environmental adaptation, generally involving the accumulation of heat-shock proteins within the cell to enhance the resistance to multiple environmental stresses **(Lou and Yousef, 1997; Wemekamp-Kamphuis *et al.*, 2004)**, moreover, secondary metabolites can be produced when they are in stress.

3. Objectives

The objective of this study was to characterize plain yoghurt fermented under various pressures at the optimal temperature of fermentation (43°C) in comparison with plain yoghurt fermented at atmospheric pressure. Additionally, the plain yoghurt was be evaluated in terms of stability during storage at 4°C. For that propose, it will be studied:

1. pH and TA
2. Syneresis
3. Microbiological counts
4. Textural characteristics
5. Colour
6. Metabolomics analyses by nuclear magnetic resonance (NMR)
7. Sugars and organic acids by high performance liquid chromatography (HPLC)
8. Total fatty acids (TFA) by gas chromatography with flame ionization detector (GC-FID)
9. Volatile compounds by gas chromatography electron ionisation mass spectrometry (GC-EI-MS)
10. Sensorial properties

With these results, it was possible to conclude about the effects of pressure during yoghurt fermentation, how different is the final product and how much it will affect during storage. In addition to this fundamental objective, if the final characteristics of the yoghurt are very different to the conventional, new food products can be obtained.

CHAPTER II – MATERIAL AND METHODS

1. Yoghurt Production

Yoghurt was produced according to the instructions provided by the inoculum manufacturer (*Iogurte Caseiro Condi 28 g*, Condi, Camarate, Portugal). One sachet of 7 g of inoculum was added to 1 litter of commercial pasteurized whole milk (*Vigor*, Lactogal Produtos Alimentares S.A, Porto, Portugal) that was purchased at a local supermarket (**Annex I - Table I-1**). The mixture was well homogenised and then was fractioned in small (5 x 4 cm, containing 10 mL in two divisors) and medium (8 x 10 cm, containing 80 mL) polyamide/polyethylene (PA/PE) bags (IdeiaPack – Comércio de Embalagens, LDA, Bodiosa, Viseu, Portugal). The bags were stored at 4ºC before fermentation for 24 h.

1.1. Yoghurt fermentation

Fermentation was carried out under different hydrostatic pressures set at 0.1, 10, 20, 30 and 40 MPa, all performed at 43°C, which is the optimal temperature of the LAB for yoghurt production **(Tamime and Robinson, 2007)**. The pH was measured with a properly calibrated pH meter for semi-solid food (Testo 205 pH, Barcelona, Spain) during the fermentation process, and the fermentation process was ended when pH value reached 4.5.

The fermentations under high pressure were performed in a lab-scale high pressure equipment (Stansted Fluid Power FPG 7100 FoodLab, Stansted, United Kingdom), using a mixture of propyleneglycol:water (40:60 v/v) as pressurization fluid, for samples fermented under 10 to 40 MPa. The HP equipment used has a pressure vessel of 2 L, and can be operated up to 900 MPa, from -20 to 110°C. Samples fermented under atmospheric pressure (0.1 MPa) were immersed in a water bath during the fermentation period. Samples of the smallest bags (10 mL) were used for microbial counts, pH measurements and syneresis evaluation at 1st, 7th, 15th and 23rd days of cold storage (4°C), while samples in the bigger bags (80 mL) were used for the texture analysis (1st and 15th day). Samples of each condition were all fermented at the same time in the high-pressure equipment and the fermentation time was determined when the final pH reached approximately 4.5 and pH was measured using an additional bag just for this purpose (80 mL). The pH was periodically measured throughout the fermentation (with measurements being carried with 30 minutes interval as the pH approached 4.5) until a pH value of 4.5 was reached. To measure the pH the pressure vessel

was decompressed and recompressed within 2 minutes time (this procedure was found to have no effect on fermentation time in previous tests (**Lopes, 2018**).

Samples in the bigger bags used for the texture analysis were transferred to small containers (20 mL), stored at 4ºC until each day of storage (7th, 15th and 23rd day) and then were frozen at -40 °C for further analysis (colour, TA, metabolomic, sugar and organic acids contain, TFA determination and quantification, volatile compounds identification) as described in **Figure I–1 (Annex I)**. Due to the limitation of the HP equipment (2 L) it was only possible to store samples up to 23 days, however the shelf life of the yoghurt is much longer. The yoghurts were stored at -40ºC after texture analysis (since texture changes with freezing). In previous tests pH was measured in fresh yoghurts and at the end of 3 months (stored at -40ºC), and the same pH was observed.

2. Microbiological analysis

LAB were determined before the milk fermentation to quantify the initial inoculum added and during storage at 4°C (1st, 7th, 15th and 23rd day) according to the method described by **Miles *et al.* (1938)**, in triplicate. To do so, yoghurt samples were ten-fold diluted in Ringer's solution, homogenized and inoculated in appropriated culture media. The viable counts of *S. thermophilus* were determined using M17 agar (Oxoid LTD, England) with lactose monohydrate (VWR Chemicals, Germany) as the culture medium, after incubation at 37°C for 24 h. Those of *L. bulgaricus* were determined using agar plates of de Man, Rogosa, and Sharpe broth (Liofilchem, Italy) with plate count agar (Liofilchem, Italy), after incubation at 37°C for 72 h. The growth of yeasts and moulds was also accessed using Rose Bengal Chloramphenicol Agar (Liofilchem, Italy), whose plates were incubated at 25°C for 5 days. In all cases, plates containing 10 to 100 CFU/0.02 mL were counted, and the counts were expressed as $\log_{10}$ CFU/mL of yoghurt. Yeasts and moulds were always below the detection limit (< 1 log CFU/mL). Measurements were done in duplicate from triplicate samples.

3. Physicochemical analyses

3.1. pH

The pH was measured during yoghurt fermentation until it reached ≈4.5 and along storage (1st, 7th, 15th and 23rd day) with a properly calibrated pH meter for semi-solid food (Testo 205 pH, Barcelona, Spain). All measurements were carried out in triplicate.

3.2. Titratable acidity (TA)

Quantification of the TA was performed with a digital burette (VWR Titras Pro 613-5287, 0-50 mL ± 0.2 %, VWR Collection, Radnor, Pennsylvania, USA) as performed by **Frye, (2013)** with some modifications: 1.50 mL of yoghurt sample were diluted in 10.50 mL of distilled water and then titrated with a 0.093 N NaOH solution that was property normalized using potassium hydrogen phthalate, until a pH 8.9 was reached (Testo 205 pH, Barcelona, Spain). The results obtained were expressed in % (mass/mass) (m/m) of lactic acid. All measurements were carried out in triplicate.

4. Syneresis, textural and colour analysis

4.1. Syneresis

Yoghurt syneresis was measured along storage, namely at the 1st, 7th, 15th and 23rd days. To do so, approximately 10 mL of sample were kept in a falcon tube, at 4°C for 24 h, and then whey that separated from samples was removed using a syringe, which was afterwards weighted. This method was adapted from **Kaminarides *et al.* (2007**). The amount of whey drained off was calculated as the syneresis index (**Equation 2**)**.** All measurements were carried out in triplicate.

$$\%\ \text{syneresis} = \frac{\text{whey weight}}{\text{sample weight}} \times 100 \qquad \textbf{Equation 2}$$

4.2. Texture

The texture of the yoghurts was assessed using a Perspex back extrusion rig comprising a cylindrical sample contained (60 mm internal diameter and 75 mm height) and a cylindrical plunger (25 mm diameter and 35 mm length), fitted to a TA.HDi texture analyser (Stable Micro Systems, Surrey, England) equipped with a 5 kg load cell. Yoghurt samples were transferred to the container (60 mL) and tested by uniaxial compression measurements, using a 20 mm compression depth at a 0.5 mm s^{-1} rate. The cylindrical probe performs a compression solicitation and forces the sample to extrude through the gap between the extrusion cylinder and the compression plunger. The Stable Micro Systems' Texture Expert Exceed software was used to extract some mechanical parameters from the force *vs*. distance curves related to the sample consistency: firmness (N), an apparent elastic modulus (N/s) and adhesiveness (mJ). Firmness was defined as the maximum force required to achieve a given deformation, being the peak force of the penetration cycle. The apparent elastic modulus was defined as the initial slope of curve of force versus time. Adhesiveness was quantified as the resistance of a substance to separate from another substance, in this case the plastic penetration probe, being the area under the negative peak. Measurements were performed in triplicate.

4.3. Colour

Samples' colour was measured with a spectrophotometer (Konica Minolta CM 2300d) at the 1st, 7th. 15th and 23rd days of storage. Calibration readings of the reference were carried out using a white plate. The samples were placed in a glass Petri dish and their colour parameters were recorded at 20 °C according to the CIE*Lab* system and directly computed through the original SpectraMagic™ NX software (Konica Minolta, Osaka, Japan), obtaining the following parameters: *L* – lightness (varying between 0 (dark) and 100 (light), *a* – degree of redness and greenness (0 to 100 (red); -80 to 0 (green)) and *b* – degree of yellowness and blueness (0 to 70 (yellow); -100 to 0 (blue)). The final colour was determined as performed by **(Liu, Hu, Zhao and Song, 2012)**, according the **Equation 3**.

$$\Delta E = ((L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2)^{1/2} \qquad \textbf{Equation 3}$$

Where $\Delta \boldsymbol{E}$ is the total colour difference between each condition and the control (yoghurt fermented at 0.1 MPa for each storage day), $\boldsymbol{L}$ and $\boldsymbol{L_0}$ are the lightness of the sample and control, respectively; $\boldsymbol{a}$ and $\boldsymbol{a_0}$ are the redness of sample and control, respectively; and $\boldsymbol{b}$ and $\boldsymbol{b_0}$ are the yellowness of sample and control, respectively. In **Annex I**, **Figure I-2,** is represented the **Equation 3** by image for better results interpretation.

5. Metabolomics analyses by NMR

One and half millilitres of yoghurt were transferred to an eppendorf (2 mL), centrifuged (at 8,000 *g* for 15 minutes, at room temperature) (Centrifuge-mixer CM-50M, ELMI Ltd., Riga, Latvia) and then filtered (white and plain membrane filter of cellulose acetate; 0.22 µm (25 mm), Advantec - Japan). The supernatant (1 mL) was then dried in a vacuum centrifuge for about 24 h. Before NMR spectral acquisition, the samples were reconstituted using 600 µL of phosphate buffer (100 mM, pH 3.0) containing 0.01 % (wt/wt) of 3-(trimethylsilyl)propionic-2,2,3,3-*d*4 acid, sodium salt (TSP-*d*4) as a chemical shift and intensity reference. The mixture was then transferred into 5 mm NMR tubes to be analysed.

^{1}H NMR spectra were recorded at 300 K on a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Germany), operating at a proton frequency of 500.13 MHz, equipped with an actively shielded gradient unit with a maximum gradient strength output of 53.5 Gcm^{-1} in a 5 mm inverse probe. For each sample, a 1D ^{1}H NMR spectrum was acquired using the noesypr1d pulse sequence (Bruker pulse program library) with water presaturation. For all spectra, 128 transients were collected into 32,768 (32 K) data points with a spectral width of 10000 Hz, an acquisition time of 3.3 s and relaxation delay of 5 s. Each free induction decay was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transformation. TopSpin 3.2 software was used to manually phase and baseline correct the spectra. The spectra were exported as a matrix, by Amix-Viewer, using R-Studio in-house scripts and subsequently normalised to TSP. The spectra were overlaid and checked in iNMR to see whether alignment was required. If required, the speaq, rolps, BiocInstaller, ChemoSpec, classyfire, gdata, ggplot2, gplots, MassSpecWavelet, matrixStats, mclust, muma, pheatmap, plyr, R.utils, RColorBrewer, reshape2, seqinr and zoo packages was used in R software. To align all peaks the baselineThresh used was 2000,

signal-to-noise ratio (SNR) Thresh was 40 and the maxshift used was 80 for all spectra, except for water zone.

5.1. Multivariate data analysis

The multivariate analysis was applied to the aligned spectra, using the ropls package (**Thévenot *et al.*, 2015**) in R software. Differences among sample groups were identified using by Pareto scaled data followed by principal component analysis (PCA). The identification of relevant metabolites was carried out by comparing the spectra with those of standard compounds from the Biological Magnetic Resonance Data Bank, the Human Metabolome Database, FooDB and the Chenomx NMR Suite software. The relative amounts of the NMR metabolites and the effect size were determined by integrating the area under the most well-separated metabolite peak using in-house R scripts.

Pairwise t-tests were carried out using the False Discovery Rate (FDR) to adjust for multiple testing. Effect sizes were calculated and corrected for small sample sizes.

6. Organic acid and sugar assessment by HPLC

Triplicate samples of yoghurt, taken at 1 and 23 days of storage, were assayed for glycolysis. One gram was added to 5 mL of 13 mmol L^{-1} sulfuric acid (H_2SO_4) and homogenized for 1 min in a vortex. The mixture was then stirred in an orbital shaker (VWR® Incubating Orbital Shaker, Model 3500I) for 30 min at 240 rpm at room temperature following another 1 min in vortex. The mixture was centrifuged (Heraeus Biofuge Stratos centrifuge, Thermo Electron corporation, Waltham, Massachusetts, United States) at 6,000 rpm for 30 min (4°C) and the supernatants were filtered through a 0.22 µm pore size membrane filter (white and plain membrane filter of cellulose acetate; 0.22 µm (25 mm), Advantec - Japan) and stored at -20°C until analysis by HPLC. The HPLC system was composed of an ion exchange Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad) maintained at 40°C and a Knauer K-2301 RI (refractive index) detector. The mobile phase used was 13 mmol L^{-1} sulphuric acid, delivered at a rate of 0.6 mL min^{-1}. The running time was 30 min and the injection volume were 30 µL.

Peaks were identified by their retention times and quantified using calibration curves prepared with the mix of the different standards (lactose, glucose and galactose for sugars and lactic, citric and formic acids for organic acids).

7. Total fatty acids (TFA) determination by GC-FID

For the analysis of the FA profile in yoghurt, triplicate samples of yoghurt, taken at 1 and 23 days of storage, were transmethylated to obtain the methyl esters of FA (FAME). About 700 mg of yoghurt were transferred to glass tubes and 200 μL of tritridecanoin (internal standard; C13) (1.7 mg mL^{-1}) were added. Then, 800 μL of hexane, 2.25 mL of methanol (MeOH) and 240 μL of sodium methoxide (5.4 M) were also added, and the mixture was homogenised by vortexing and heated at 80°C for 10 minutes. The tubes were cooled in ice, and 1.25 mL of N,N-dimethylformamide (DMF) and 1.25 mL of H_2SO_4/MeOH (3 M) were added, vortexed and heated at 60°C for 30 min. The mixture was again cooled in ice, and 1 mL of hexane was added, homogenised by vortexing for 30 s and centrifuged for 5 min at 1,250 *g* at 18°C. The upper layer of the resulting solution was collected for further GC-FID analysis.

The GC-FID used in FAME analysis was composed of a gas chromatograph HP6890A (Hewlett-Packard, Avondale, Pennsylvania, USA), a flame-ionization detector (GC-FID) and a BPX70 capillary column (60 m × 0.25 mm × 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). Hydrogen was used as the carrier gas at 20.5 psi, the injector temperature was 250°C, the injection volume was 1 μL (25:1 split) and the FID detector temperature was 275°C. The oven temperature program was as follows: 60°C (held 5 min), then raised at 15°C/min to 165°C (held 1 min) and finally at 2°C/min to 225°C (held 2 min). For the individual identification of fatty acids, Supelco 37 and FAME from CRM-164 were used. Also, calculation of response factors and detection and quantification limits (LOD: 0.79 μg FA/mL; LOQ: 2.64 μg FA/mL) were assayed with GLC-Nestlé36 protocol, as used by Universidade Católica do Porto (UCP) (Escola Superior de Biotecnologia (ESB)).

Fatty acids were quantified through the correlation of the area of the internal standard with the corresponding concentration, and assuming the same response for each individual fatty acid.

7.1. Nutritional (lipidic) quality indices

There are several indices to be used as indicators for determining whether a diet is atherogenic or promotes coronary heart diseases (CHDs) (**Chalabi *et al.*, 2018**). Based on the FA composition, the atherogenicity and thrombogenicity indices were calculated. The index of atherogenicity (IA) indicates the relationship between C12, C14, and C16 (pro-atherogenic factor) and unsaturated FA (USFA). In this regard, the **Equation 4** was applied, similarly to **Chalabi *et al.* (2018), Naydenova *et al*, (2014), Senso *et al.* (2007)** and **Ulbricht and Southgate, 1991**).

$$IA = \frac{C12+(4\times C14)+C16}{\sum MUFA+PUFA_{n-6}{}^{*}+PUFA_{n-3}{}^{*}} \qquad \textbf{Equation 4}$$

*n-6 and n-3 are respectively FA omega-6 and omega-3, MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids)

The ratio of C14, C16, and C18 (pro-thrombogenetic) to USFAs (anti-thrombogenetic) is described as the index of thrombogenicity (IT). This index refers to the tendency for clot formation in the blood vessels. The IT value was calculated according **Equation 6**:

$$IT = \frac{C14+C16+C18}{(0.5\times\sum MUFA+0.5\times PUFA_{n-6}+3\times PUFA_{n-3})+\frac{PUFA_{n-3}}{PUFA_{n-6}}} \qquad \textbf{Equation 5}$$

*n-6 and n-3 are respectively FA omega-6 and omega-3, MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids)

Other indicators included the ratio of omega-6/omega-3, monounsaturated fatty acids (MUFA)/ polyunsaturated fatty acids (PUFA), and the PUFA to saturated fatty acids (SFA) ratios were also calculated.

8. Volatile compounds extraction by SPME and identification by GC-EI-MS

Volatile compounds profile was determined by headspace-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS) (GC456 MS SCION TQ) (Bruker, Bremen, Germany). Initially 5 g of each sample, in triplicate for 1st and 23rd days of storage, were placed in 20 mL headspace vials, then 5µL of cyclohexanone (98 %) (93.1 mg/L) was added as internal standard. The vials were heated at 60°C for 20 min with a constant stirring (250 rpm). After that, the SPME fiber coated with divinybenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm; 1 cm, Supelco Inc.) was exposed in each sample for 40 min, still at 60°C, for volatiles absorption.

Volatiles were thermally desorbed for 5 min in the injector (splitless mode for 1 min; 250°C) of the GC-MS system. Chromatographic separation was performed on a fused-silica CP-Wax 58 FFAP Capillary GC column (50 m × 0.25 mm I.D × 0.20 µm film thickness, Agilent J&W GC columns) with a temperature program starting at 40 °C during 10 min than 2ºC/min until 250°C for 5 min (total run time of 120 min). The MS transfer line and ion source were both set to 250°C, electron ionization (EI) of 70 eV; set in full scan mode (m/z 35 to 500 with a scan time of 1000 ms). Compounds were identified by comparing the respective mass spectra with a mass spectral database (NIST v2.2, nist.gov and by comparing of other articles), moreover acetaldehyde, acetone and ethanol were used as standards to confirm the identifications.

As the analysis of volatile compounds was performed only in July, due to the availability of the CINATE (UCP-ESB), only the main compounds identified, and the main differences observed in the chromatograms are described, since there was no time to quantify the compounds to deliver the thesis on time.

9. Sensorial analyses

Preliminary the samples were evaluated in a blind test by a non-trained panel of eight (8) usual yoghurt consumers. The panel was given a yoghurt fermented under atmospheric pressure and another fermented under pressure (all identified by letters code). The sensory panelists evaluated them relatively to the texture, odour and flavour by an order of preference. At the end, the panellists identified their favourite yoghurt, as shown in the sensorial analysis sheet (**Annex I – Figure I-3**).

10.Statistical analysis

The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %, to infer statistical differences/similarities between conditions and storage days. For this, it was defined that different upper-case letters in tables and figures indicate statistically significant different ($p < 0.05$) values for a given day of storage at different fermentation pressures, while lower-case letters indicate statistically significant different ($p < 0.05$) values for different days of cold storage at a fermentative pressure. All the performed analyses were done in triplicate and all these values were counted for the statistics on pressure variation and storage day.

CHAPTER III – RESULTS AND DISCUSSION

THE FIRST RESULTS PRESENT IN THIS SECTION, UNTIL SECTION 5. TEXTURE, WERE PUBLISHED IN AN ARTICLE, WHICH TITLE IS “A MICROBIOLOGICAL, PHYSICOCHEMICAL, AND TEXTURE STUDY DURING STORAGE OF YOGHURT PRODUCED UNDER ISOSTATIC PRESSURE”

1. Yoghurt Fermentation

The fermentation time of yoghurt was determined as time to reach the final pH of 4.5, which is the usual criterion used in industry to stop the fermentation by cooling. The results obtained indicate a linear relationship between the fermentation time (FT) and pressure (PF) (**Annex II – Figure II- 1**): $tF\ (h) = 9.05 \times 10^{-2}\ PF\ (MPa) + 4.51$ ($R^2 = 0.965$).

In fact, with the increase of pressure, the fermentation time also increased. This is not surprising as hydrostatic pressure is reported to slow down microbial metabolism **(Mota *et al.*, 2015**), as a possible response to the sub-lethal pressures at which the fermentation process occurred.

2. Microbiological analyses

Viable bacterial counts were determined after milk inoculation (before fermentation) and revealed values of 7.09 ± 0.11 and 7.01 ± 0.01 $\log_{10}$ CFU/mL for *S. thermophilus* and *L. bulgaricus,* respectively. This represents a ratio of ≈1:1 which is recommended by the current literature for a good synergistic effect **(Hill and Kethireddipalli, 2013**). During the storage period, *S. thermophilus* and *L. bulgaricus* were also enumerated and the results are shown in **Figure 7 (A)** and **(B)**, respectively. Yeasts and moulds were not detected along the whole storage period.

S. thermophilus counts in yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa), showed differences for the 1st day between yoghurts fermented at lower and higher pressures. Yoghurt fermented at 0.1, 10, 20 and 30 MPa presented higher ($p < 0.05$) microbial populations than the one fermented at 40 MPa.

These differences were less evident along storage, and no statistical differences ($p > 0.05$) were observed between samples at 7th and 15th day of refrigerated storage. By the 23rd day, all yoghurts fermented under pressure presented similar ($p > 0.05$) *S. thermophilus* counts, which were similar ($p \geq 0.05$) to the samples fermented at 0.1 MPa (except for yoghurt fermented at 0.1 MPa).

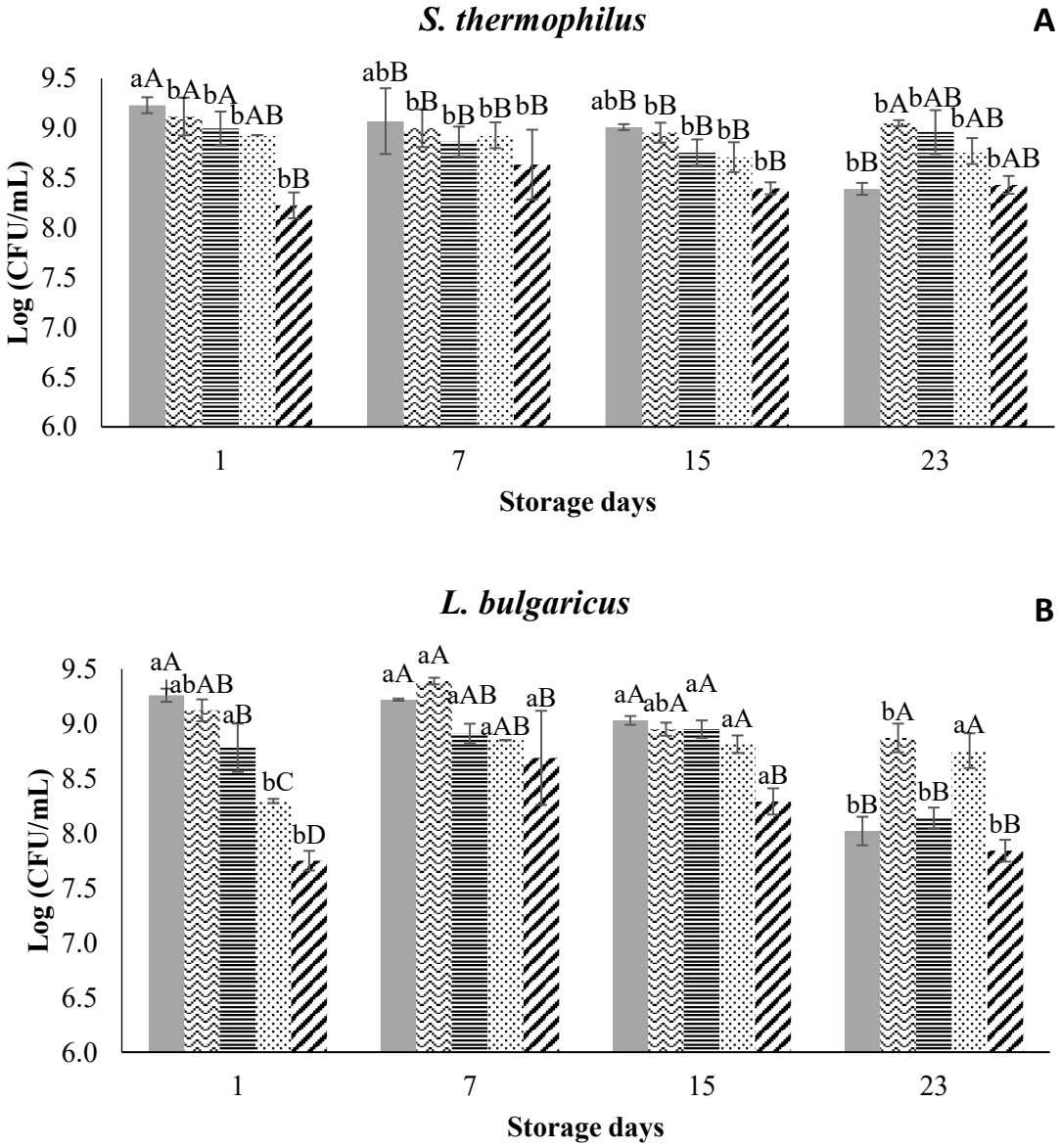


Figure 7 – Lactic acid bacteria counts (Log_{10} CFU/mL) during yoghurt storage time at 4°C fermented under different pressures (0.1 MPa (▩); 10 MPa (▨); 20 MPa (≣); 30 MPa (⁘); 40 MPa (▨). **(A)** *S. thermophilus* count and **(B)** *L. bulgaricus* count. Different lower (a-b) and upper (A-D) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Generally, *L. bulgaricus* population decreased ($p < 0.05$) from the 1st to the 23rd days of storage regardless the fermentation pressure, with exception for yoghurts fermented under 30 and 40 MPa, whose populations were higher ($p < 0.05$) and similar ($p > 0.05$) by the 23rd day of storage (compared with the 1st day), respectively. Similarly, to that observed

for *S. thermophilus*, at the 1st day, *L. bulgaricus* counts were lower ($p < 0.05$) as the pressure increased when compared with control experiment. These differences in counts depending on the level of pressure applied were also less pronounced along storage: the only difference after 15 days was observed between the sample fermented at 40 MPa and the remaining ones ($p > 0.05$).

Although the amount of LAB did not vary significantly ($p > 0.05$) with increasing pressure for the same day of storage, there was a linear-like tendency for its decrease with pressure increment used for fermentation (**Annex 2**, **Figure II-2 (A)** and **(B)**). In fact, the higher is the pressure applied in a food, more the higher affected is the microorganism growth (**Mota *et al.*, 2015**). These observations are in accordance with our results; however, it seems that when yoghurt returns to atmospheric pressure, during cold storage, LAB appear to be more active than those that ferment at atmospheric pressure and possibly more physicochemical changes may occur during this time.

Despite that the results for the control samples (fermentation performed at 0.1 MPa) were similar to those reported by **Salvador and Fiszman (2004)**, we did not observe a faster decrease of the *L. bulgaricus* counts than of those for *S. thermophilus* as reported by these authors. Worth to mention that along the 23 days of cold storage and for the different fermentation conditions studied, the total counts for these microorganisms were higher than 10^7 CFU/g, which is in accordance with the recommendations from the Codex Alimentarius (**Codex Alimentarius International Food Standards, 2003**).

3. Syneresis, pH and TA

Syneresis did not change significantly ($p > 0.05$) along storage, for each fermentation pressure. Generally, pressure had a significant effect ($p < 0.05$) on syneresis. Yoghurts fermented at 0.1 and 10 presented similar ($p > 0.05$) syneresis values, while at 20, 30 and 40 MPa, the syneresis was considerably higher ($p < 0.05$) (**Table 13**). **Salvador and Fiszman (2004)** observed that when whole yoghurt was stored at 10°C, syneresis increased up to the 40th day, but then stabilized. In this case, the storage temperature was lower and that may be the reason why this parameter did not change significantly along storage. Furthermore, **Lopes *et al.*, (2019)** also concluded that when yoghurt was fermented under higher pressure (30 MPa), more syneresis was observed at the end of fermentation.

The pH variation during storage (1st, 7th, 15th and 23rd days) is represented in **Table 13**. At each analysed storage time (1, 7, 15 23 days), samples fermented at different pressures showed similar pH values ($p > 0.05$). With refrigerated storage, the pH of yoghurts fermented at 0.1, 20 and 40 MPa decreased ($p < 0.05$) between 1st and 15th day and then remained unchanged ($p > 0.05$) until the end of the experiments. pH value slightly decreased ($p > 0.05$) in yoghurts fermented at 10 MPa until 15th but more pronounced differences ($p < 0.05$) were evident between 7th and 23rd day of storage. Contrarily, yoghurts fermented under 30 MPa faced a slight pH decrease ($p > 0.05$) until the 23rd day. The pH along storage showed a linear trend to decrease (1st to 23rd day) for pressurized yoghurts ($R^2 > 0.89$), however, in control samples this trend is not so remarkable ($R^2 = 0.86$), as seen in **Annex II – Table II-1**.

TA showed no statistical significance ($p > 0.05$) according to the applied pressure (**Table 13**). These results are in accordance with **Salvador and Fiszman, (2004)** that observed no differences in acidity during storage (10°C) of whole yoghurt fermented at atmospheric pressure. These results are contrary to those observed by **Mota *et al.* (2015)**, who reported that the TA was considerably lower for yoghurts produced under pressure. In the cited study, the fermentation process occurred for 600 min, regardless the final pH, while in the present study, the fermentation was carried out until a final pH of 4.5 was reached, and that may be a possible reason to explain these differences.

The results discussed in this section show that pH tends to decrease slightly along the storage time for all pressure applied, but TA is maintained in all yoghurts throughout the storage. Yoghurts fermented at 20 and 40 MPa showed more syneresis than other conditions and this difference was maintained during storage for all samples.

Table 13 - pH, titratable acidity (TA, % (m/m) of lactic acid) and syneresis (%) variation (± standard deviation) for yoghurts fermented at each condition (0.1, 10, 20, 30 and 40 MPa, for 1, 7 15 and 23 days during cold storage).

Storage day vs Fermentation Pressure (MPa)	1	7	15	23	Parameter
0.1	4.49 ± 0.02^{aA}	4.42 ± 0.01^{abA}	4.37 ± 0.05^{bB}	4.37 ± 0.04^{bA}	pH
10	4.54 ± 0.03^{aA}	4.50 ± 0.02^{aA}	4.41 ± 0.03^{abAB}	4.40 ± 0.02^{bA}	
20	4.57 ± 0.03^{aA}	4.50 ± 0.01^{abA}	4.42 ± 0.02^{bAB}	4.41 ± 0.02^{bA}	
30	4.54 ± 0.00^{bA}	4.48 ± 0.00^{bA}	4.48 ± 0.02^{bA}	4.43 ± 0.04^{bA}	
40	4.54 ± 0.02^{aA}	4.52 ± 0.00^{abA}	4.45 ± 0.03^{bAB}	4.44 ± 0.02^{bA}	
0.1	0.76 ± 0.06^{aA}	0.84 ± 0.04^{aA}	0.91 ± 0.03^{aA}	0.86 ± 0.04^{aA}	TA %(g/g) lactic acid
10	0.89 ± 0.11^{aA}	0.79 ± 0.03^{aA}	0.82 ± 0.02^{aA}	0.76 ± 0.03^{aA}	
20	0.78 ± 0.00^{aA}	0.78 ± 0.00^{aA}	0.80 ± 0.01^{aA}	0.83 ± 0.04^{aA}	
30	0.96 ± 0.02^{aA}	0.93 ± 0.09^{aA}	0.85 ± 0.03^{aA}	0.92 ± 0.10^{aA}	
40	0.82 ± 0.03^{aA}	0.83 ± 0.03^{aA}	0.90 ± 0.01^{aA}	0.85 ± 0.05^{aA}	
0.1	0.00 ± 0.00^{aA}	3.37 ± 0.33^{aA}	4.17 ± 1.17^{aA}	6.38 ± 1.88^{aA}	% Syneresis
10	3.57 ± 1.21^{aA}	5.28 ± 0.31^{aA}	3.53 ± 0.64^{aA}	5.47 ± 0.25^{aA}	
20	20.95 ± 3.10^{aB}	18.91 ± 5.16^{aB}	18.72 ± 5.44^{aAB}	22.85 ± 7.64^{aB}	
30	18.07 ± 5.54^{aB}	27.53 ± 1.86^{aBC}	25.44 ± 1.84^{aB}	27.07 ± 1.29^{aB}	
40	44.24 ± 2.96^{aC}	38.34 ± 6.88^{aC}	40.92 ± 11.22^{aB}	43.15 ± 3.08^{aC}	

Different lower (a-b) and upper (A-C) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 % and all analyses were done in triplicate (n=3).

4. Colour

Table II-2, present in **Annex II**, reports the changes in the *L*, a** and *b** colour parameters of yoghurt observed during the 23 days of storage. Overall no colour variations ($p > 0.05$) were observed between yoghurts fermented under pressure and the control (fermented at atmospheric pressure), regardless the fermentation pressure and sampling day, suggesting that the obtained yoghurts retained their characteristic colour, except for the *L** that showed an increment with pressure for 23 days of storage up to 30 MPa.

5. Texture

Texture was another parameter analyzed in this work, since it is one of the main characteristics that defines yoghurt quality and consumer acceptance. **Figure 8** represents yoghurt firmness (N) at 1st and 15th day of storage. There seems to be a trend in firmness to increase as the fermentation pressure increase, which becomes more evident along refrigerated storage. The firmness parameter was statistically similar ($p > 0.05$) for all the fermentation conditions at the 1st day of refrigerated storage, with exception for yoghurt produced under 30 MPa. After 15 days of cold storage, the firmness increased ($p < 0.05$) for all yoghurts (except for those produced at 0.1, 10 and 30 MPa), and those fermented under 40 MPa presented the highest ($p < 0.05$) firmness. These results do not match those obtained by **Lopes *et al.*, (2019)**, who found that yoghurts produced under pressure (10 and 30 MPa) presented lower firmness levels than those produced at atmospheric pressure (0.1 MPa). This might be due to the fact that in the present study pasteurized whole milk was used, while in the work of **Lopes *et al.*, (2019)**, reconstituted whole milk powder was used. As can be seen in **Figure II-3**, **Annex II**, there was a tendency for the firmness to increase with increasing fermentation pressure or increasing storage time.

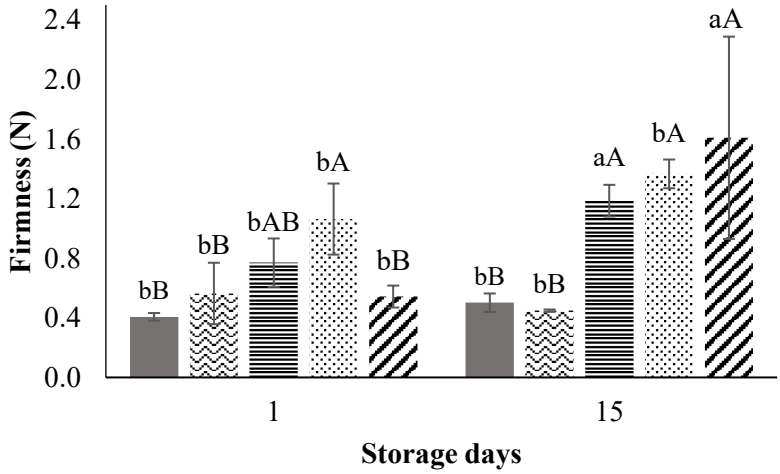


Figure 8 - Firmness (N) of yoghurt produced under pressure (10 MPa (≈); 20 MPa (≡); 30 MPa (⁙); 40 MPa (▨)) and at atmospheric pressure(0.1 MPa (■)) measured at 1st and 15th day of cold storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

When it comes to the apparent elastic modulus, values seemed to decrease with the fermentation pressure increase, as this parameter was significantly lower ($p < 0.05$) for yoghurts produced under 20, 30 and 40 MPa compared to yoghurt produced at 0.1 MPa (**Figure 9**). Cold storage seems not to affect this parameter, as no statistical differences $p > 0.05$) were observed between the 1st and 15th days of storage within each fermentation pressure.

This behaviour is clearly seen in **Figure II-4** (**Annex II**) where a linear decreasing tendency is observed. The elastic modulus showed no variation with storage. In fact, the lower the plasticity (elastic modulus) of a product is, the greater its firmness, which is in accordance with the results obtained.

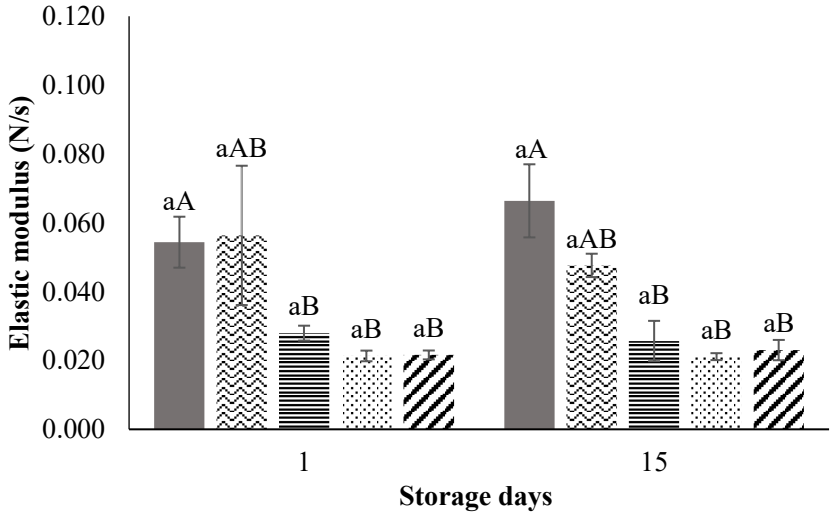


Figure 9 - Apparent elastic modulus (N/s) of yoghurt produced under pressure (10 MPa (▨); 20 MPa (▤); 30 MPa (▩); 40 MPa (▨)) and at atmospheric pressure (0.1 MPa (■)) measured at 1st and 15th day of cold storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Values of adhesiveness are shown in **Figure 10**. The observed values for pressures between 20 and 40 MPa were very low and close to the instrument resolution. Adhesiveness of yoghurts fermented at 0.1 and 10 MPa did not present significant differences ($p > 0.05$) along storage and for the same storage day with pressure (**Figure**

10). Overall, it is possible to conclude that with the increase of pressure during fermentation, yoghurt is less stiff and adhesive, but firmer.

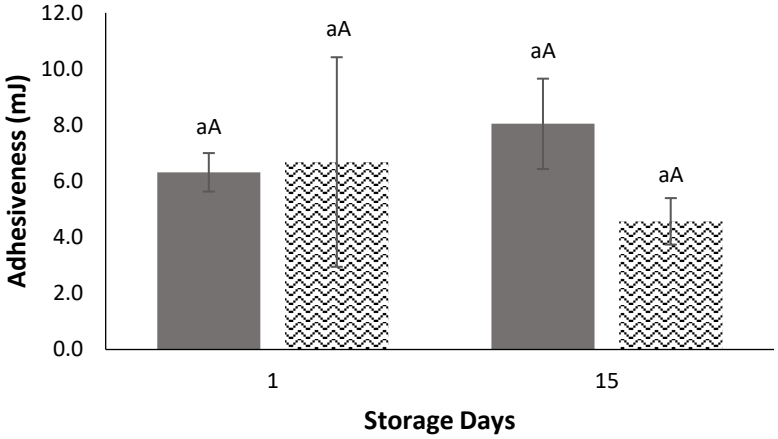


Figure 10 - Adhesiveness (mJ) of yoghurt produced under pressure (10 MPa (▒)) and at atmospheric pressure (0.1 MPa (■)) measured at 1st and 15th day of cold storage. Similar lower (a) and upper (A) case letters indicate statistical similarities ($p > 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

6. Metabolomics analysis by NMR

An example of the characteristic 1D ^{1}H NMR spectra of the yoghurt samples fermented under 0.1, 10, 20, 30 and 40 MPa are shown in **Figure 11 A** for the 1st day of storage. The principal peaks are identified and described in **Table 14**, however it was difficult to separate the different sugars namely lactose, glucose and galactose because they have peaks in common, so the sugar peaks are the sum of galactose, lactose and glucose content/signal, and were divided into nine sub-groups.

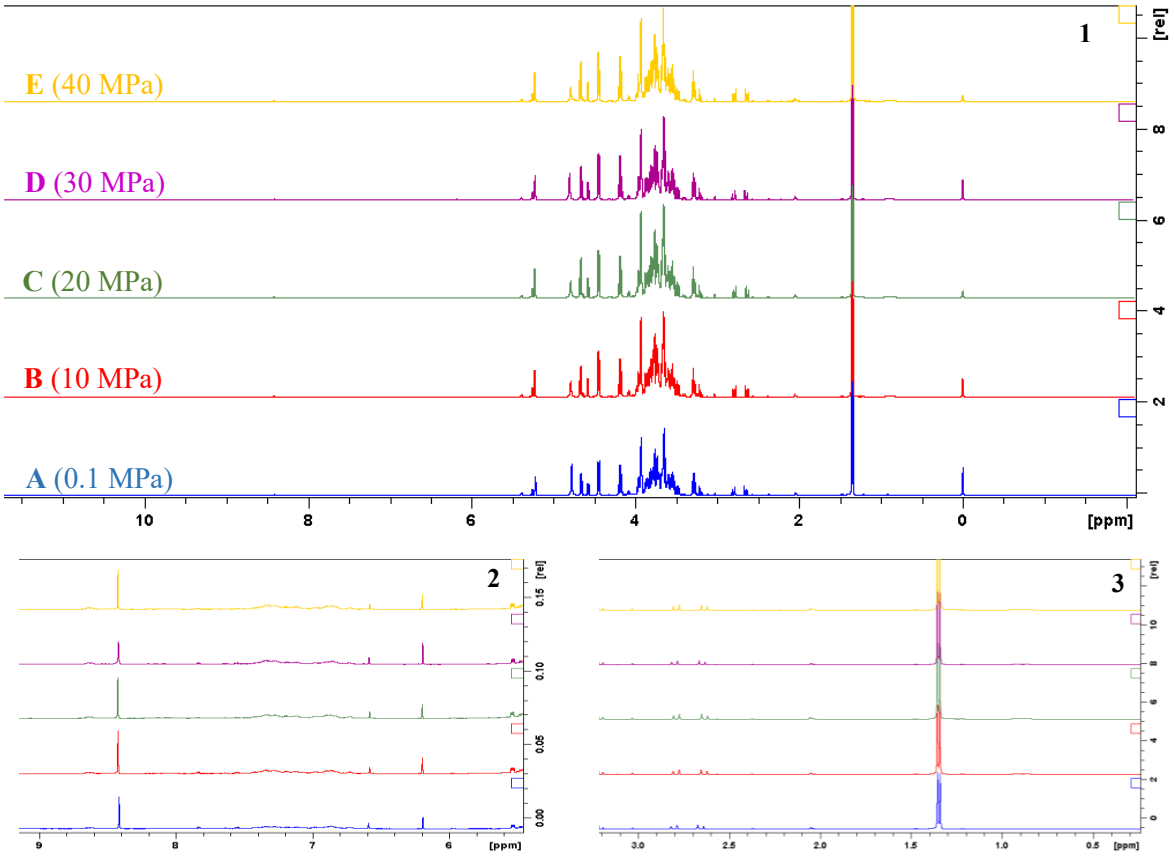


Figure 11 - 500 MHz 1H NMR spectra of yoghurt produced under different pressures 0.1 (A) (blue spectra), 10 (B) (red spectra), 20 (C) (green spectra), 30 (D) (purple spectra) and 40 (E) (yellow spectra) MPa at 43ºC: **(1)** full spectra; and expansions for **(2)** aromatic region (5.8 – 9.0 ppm) and **(3)** aliphatic region (0.5 – 3.1 ppm)

In order to identify some of the metabolites present in the yoghurt samples, spectral comparisons with databases was performed as described in **Material and Methods** section (page 51). Regarding the full spectra of the different yoghurts (**Figure 11 A**) no obvious differences could be seen. The peaks with higher intensity correspond to lactate and sugars,

namely lactose and galactose. These results are in accordance with the directed analysis for sugars and organic acids that will be described below (page 75). Minor compounds could also be observed in the aromatic (5.8 – 9.0 ppm) and aliphatic (0.5 – 3.1 ppm) regions.

For instance, the aromatic region (**Figure 11 B**) is characterized by the presence of peaks corresponding to formate, aromatic amino acids, such as phenylalanine, histidine, tryptophan and tyrosine (6.8 – 8.0 ppm), and other peaks that could not be assigned (e.g. 6.19 and 6.58 ppm). The aliphatic region (**Figure 11 C**) is characterized by peaks corresponding to organic acids, alcohols and aliphatic amino acids, the main products of fermentation, including lactate, citrate, acetate, pyruvate, acetoin, 2,3-butanediol, diacetyl, among others. In these cases, the differences observed between samples were not as pronounced as for the aromatic region, but different intensities were obtained for peaks identified as 2.3-butanediol, acetate, acetoin, diacetyl and for unknown_2.

Table 14 - List of the principal metabolites identified in samples by comparison with databases and an appropriate software (Chenomx), with the respective chemical shifts.

Compounds	Chemical shift (ppm)	Compounds	Chemical shift (ppm)
2,3-butanediol	1.12 – 1.16	Sugars_1	3.10 – 4.10
Acetate	1.87 – 1.95	Sugars_2	4.42 – 4.48
Acetaldehyde	2.03 – 2.08	Sugars_3	4.56 – 4.60
Acetoin	2.21 – 2.24	Sugars_4	4.62 – 4.70
Citrate	2.60 – 2.85	Sugars_5	4.76 – 43.82
Diacetyl	2.37 – 2.38	Sugars_6	5.21 – 5.245
Formate	8.41 – 8.43	Sugars_7	5.25 – 5.29
Lactate	1.24 – 1.28; 4.14 – 4.22	Sugars_8	5.36 – 5.455
Pyruvate	2.55 – 2.60	Sugars_9	6.185 – 6.20
Alanine	1.46 – 1.49	Unknown_1	0.75 – 1.00
		Unknown_2	3.02 – 3.05

In order to identify the differences observed for samples fermented under different pressure conditions, a PCA was carried out using a dataset generated from the full ^{1}H NMR spectra. PCA is an unsupervised statistical analysis that is widely used as a first exploratory

step in metabolomics studies. This statistical tool converts high dimensional data into fewer dimensions, maintaining as much variance from the original data as possible (**Boccard *et al.*, 2010; Nyamundanda *et al.*, 2010**). As a result, sample distribution in the principal component (PC) space is given by score plots, where the Euclidian distance between individual samples reflects the degree of the variation in metabolite profiles among samples and the loading plots describe the contribution of individual metabolites to each PC (**Sugimoto *et al.*, 2012**). The scores plot obtained in this work is presented in **Figure 12**. The PCA model showed a good fit (R2X = 0.74), with the first and second principal components (PC1 (t1) and PC2 (t2)) explaining 48 and 14 % of the total variance, respectively.

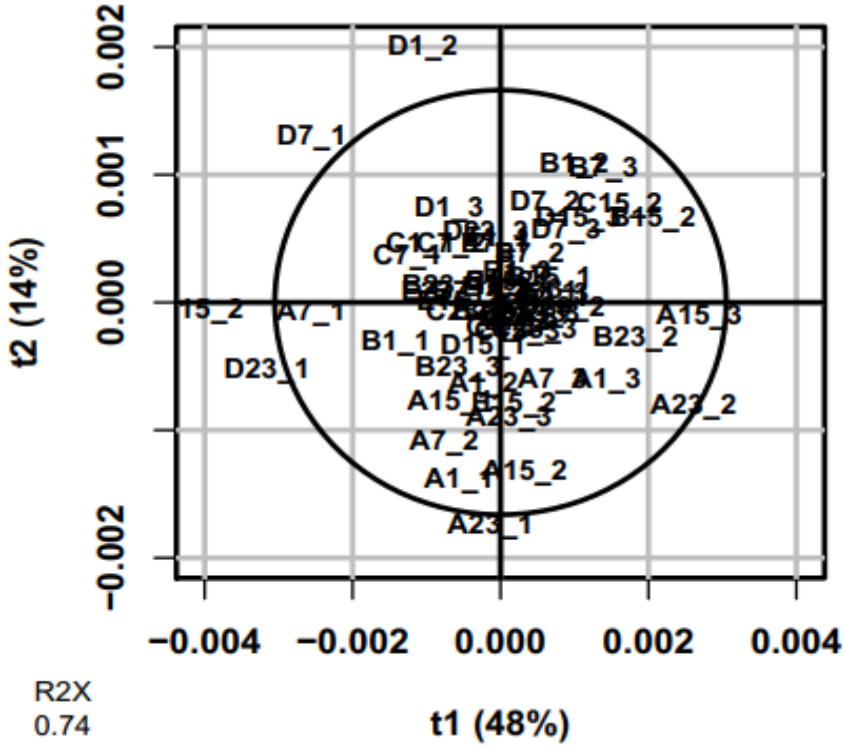


Figure 12 - PCA scores plot of yoghurt produced under different conditions of pressure (0.1, 10, 20, 30 and 40 MPa) obtained by 1D ^{1}H NMR. *Legend of sample name code:* Letters represent the pressure of fermentation A to E means 0.1 to 40 MPa, the first number at the right of letter mean the day of storage (1, 7, 15 or 23) and the second number represent the number of replica (1, 2 or 3).

The PCA scores plot revealed no significant and clear separation between the control samples (A – fermented under 0.1 MPa), samples subjected to pressure (B, C, D and E – fermented under 10, 20, 30 and 40 MPa) and sample storage time (1, 7, 15 and 23). However, the reproducibility between triplicates was in some cases of the order of the group separation,

as can be seen in **Figure 12**. Furthermore some outliers were identified, namely A7_1, A15_2, A23_1, D1_2, D7_1, D15_2, D23_1 and E23_3 (Letters represent the pressure of fermentation A to E means 0.1 to 40 MPa, the first number at the right of letter mean the day of storage (1, 7, 15 or 23) and the second number represent the number of replica (1, 2 or 3), as can be confirmed the PCA of different yoghurts for each one day of storage is compared (**Annex II – Figure II-5**). However, when deleted, other outliers can be seen (**Annex II– Figure II-6**), indicating that all samples have similar composition. For that reason, all outliers (one replica of some described samples) were maintained for further analysis.

Analysing the loading plots (**Figure 13**) indicated that sugars are the main metabolites that positively contributed to PC2, while lactate is the main metabolite that negatively contributed. On the other hand, both sugars and lactate positively contributed to PC1, which are the main responsible metabolites for samples separation.

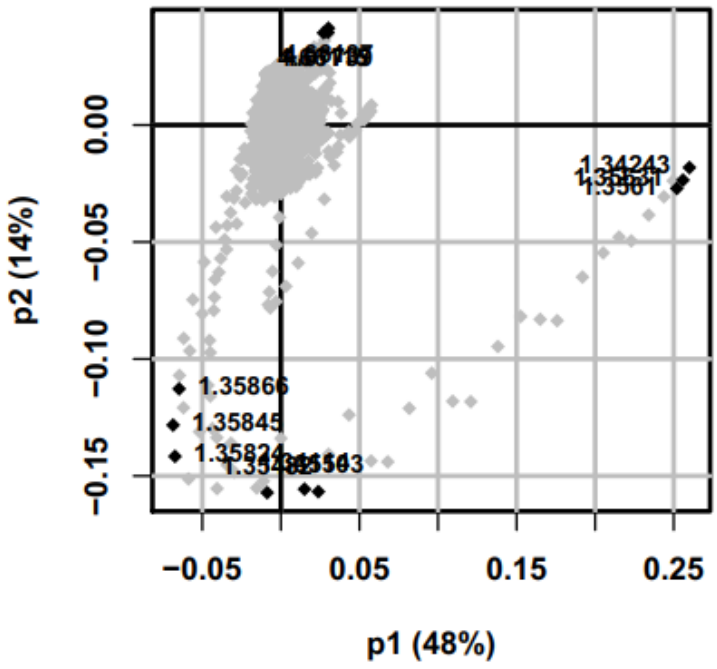


Figure 13 – Loading plot showing the metabolites, by its chemical shift (ppm), that contribute positively or negatively to PC1 (p1) and PC2 (p2).

This means that samples showing higher PC1, contain different amounts of lactate and sugars and the samples showing higher PC2, contain also different amounts of sugars. However, both PC1 and PC2 presented lower contributions to sample discrimination,

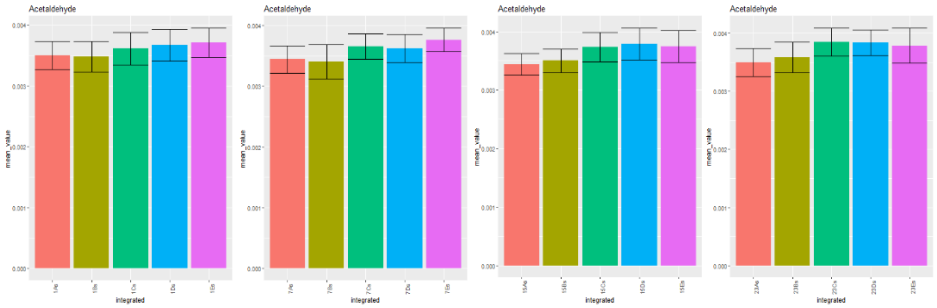
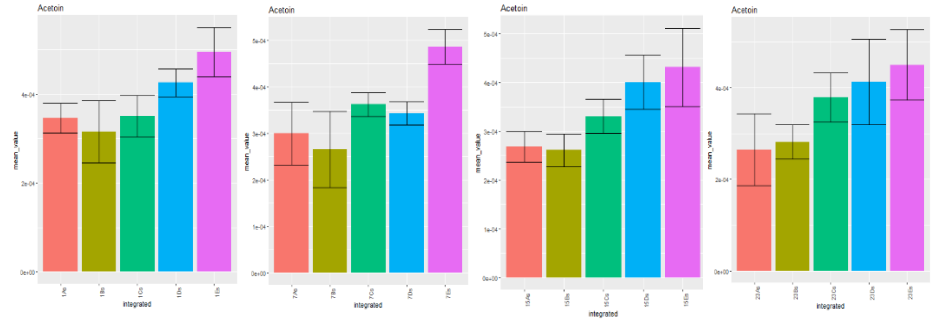
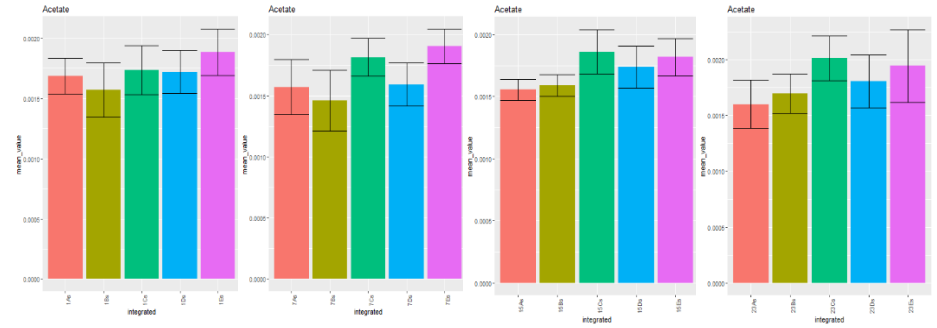
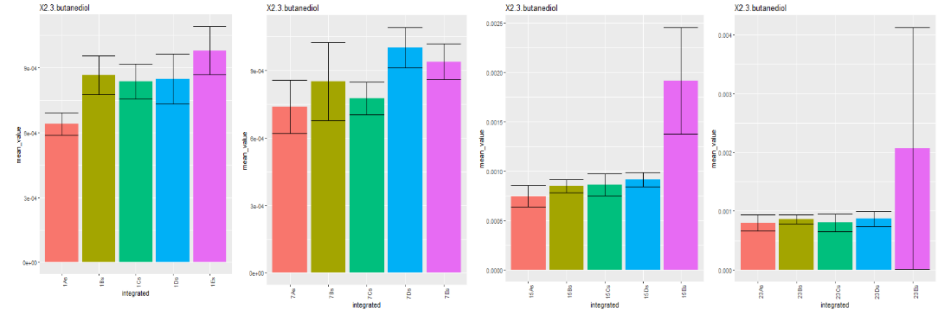
making difficult to disclose the differences between the metabolic profile of the different samples. Thus, this model had low significance, which may be due to high similarity between samples.

In order to semi-quantitatively compare the compositional changes between the yoghurt samples analysed, normalized areas of the compounds identified were calculated. Firstly, the identification of the signals corresponding to the metabolites present in the yoghurt samples was performed. The identification of different sugars was impossible due to the overlap of several signals in the sugar region, however other important yoghurt components were successfully identified, such as lactate, citrate, formate, pyruvate, diacetyl, acetoin, acetaldehyde, acetate, alanine, and 2,3-butanediol. Several unknown metabolite peaks were also observed.

As mentioned previously, in addition to lactate production, starter cultures can also produce several compounds in lower amounts that are responsible for yoghurt flavour. In these cases, pyruvate is used as a metabolic precursor of the mixed acid metabolism. By analysis of the spectra, signals corresponding to some of these compounds were identified, including pyruvate, acetate, formate, acetaldehyde, diacetyl, acetoin and 2,3-butanediol.

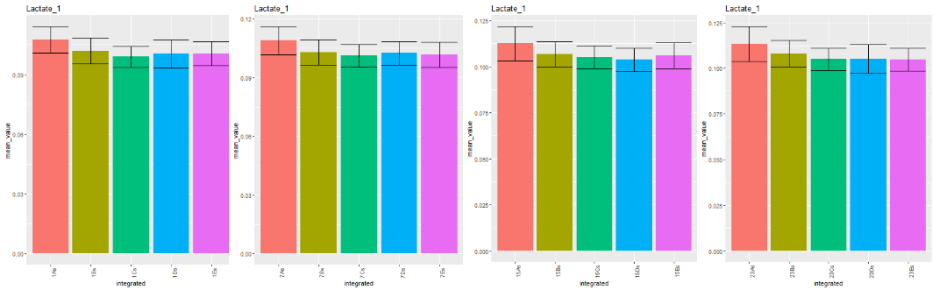
No statistical differences ($p < 0.05$) were verified between the content of each compound (namely acetaldehyde, acetate, diacetyl, lactate, alanine, sugars, pyruvate and the unknown compounds) along yoghurt storage, except for 2,3-butanediol that increases between the 7^{th} and 15^{th} day of storage for yoghurt fermented under 40 MPa. Generally, there were no statistical differences ($p < 0.05$) between the content of compounds in yoghurts fermented under different pressures, as seen for acetaldehyde, acetate, lactate, alanine, pyruvate and sugars, except for 2,3-butanediol, acetoin, diacetyl and formate. The normalized areas of these compounds are presented in **Table 15**, except for sugars and the unknown compounds.

Table 15 - Metabolite plots showing the accumulation of each compound for yoghurts fermented under pressure: 0.1 (red columns), 10 (yellow columns), 20 (green columns), 30 (blue columns) and 40 MPa (purple columns) during yoghurt storage.

Compound	1st day	7th day	15th day	23rd day
Acetaldehyde				
Acetoin				
Acetate				
2,3-butanediol				

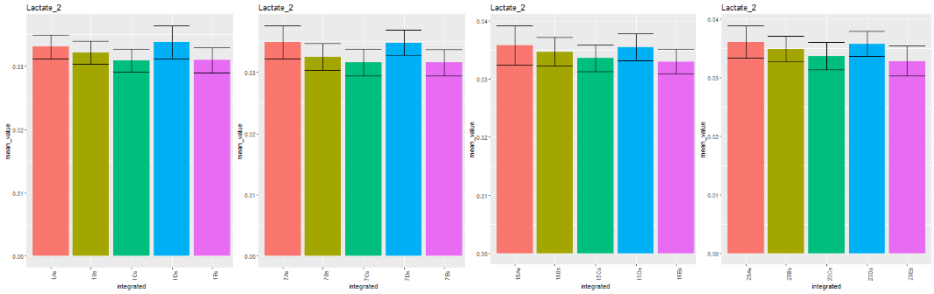
Lactate

(1.24–1.28 ppm)

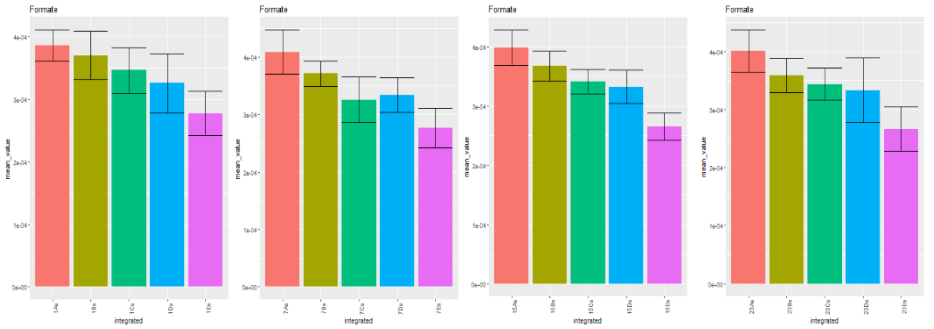


Lactate

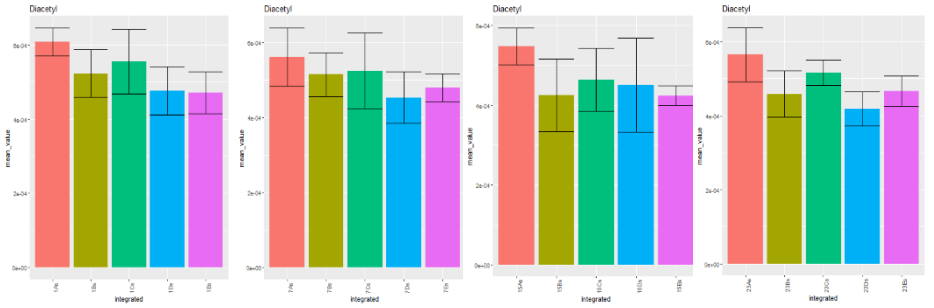
(4.14-4.22 ppm)



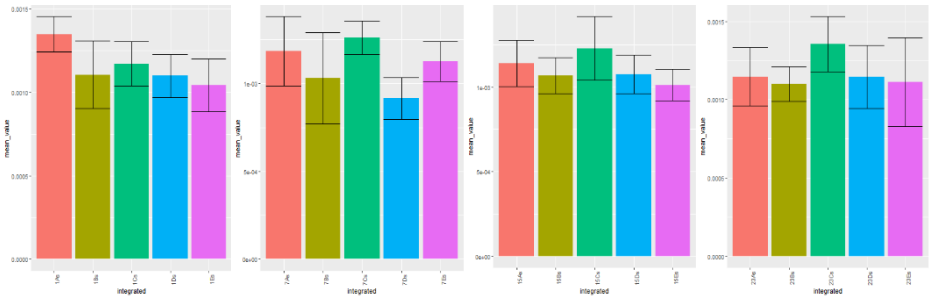
Formate



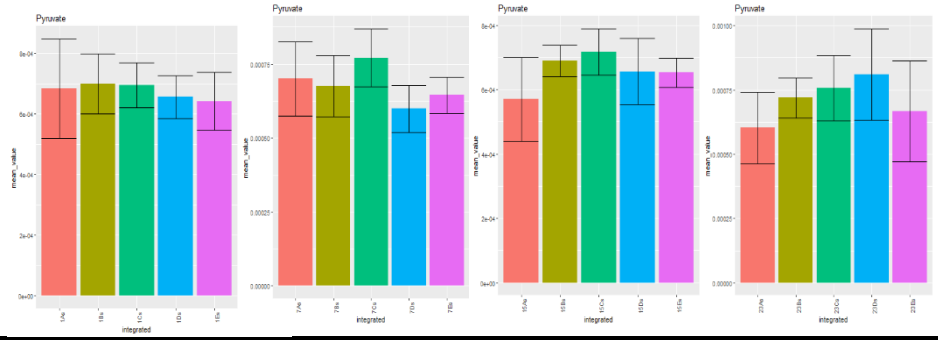
Diacetyl



Alanine



Pyruvate



The compounds that contribute to the taste and aroma of yoghurt varied in terms of relative abundance between the samples. Acetoin showed different abundances between the yoghurt fermented under 40 MPa (E) and the control (fermented under 0.1 MPa (A)). On the other hand, in all analysed days, acetoin was more abundant in the yoghurts fermented under 20, 30 and 40 MPa, but it was observed a diffrence between acetoin and diacetyl and formate, the last ones are more abundant in the control yoghurt samples.

The abundance of 2,3-butanediol compound is lower in the control sample for the first day of storage when compared with the other samples. However, its content seems to increase on the 7th day of storage and is then stabilizes until the 15th day for all samples, except for the fermented under 40 MPa that increase their 2,3-butanediol content.

As mentioned before, both diacetyl and acetoin are important for the typical yoghurt aroma, being responsible for the butter-like flavour. The production of these two compounds is linked, since acetoin is the reduced form of diacetyl, produced with the irreversible action of diacetyl reductase. Therefore, the fermentation conditions used during this work may have affected the activity of diacetyl reductase, when higher pressures cause an activity increase, due to the higher acetoin levels observed in the samples fermented with higher pressure. The same conclusion can be applied to acetoin reductase that reduce acetoin in to 2,3-butanediol. In the other hand the abundance of acetaldehyde is similar for all samples, which may suggest that the enzyme diacetyl synthase is not affected (positively) by pressure, so diacetyl and acetoin are formed by α-acetolactate (derived from pyruvate) and, possibly, pressure also active acetolactate decarboxylase.

The results obtained by the analysing spectra from 1D ^{1}H NMR was a pertinent approach to understand how different the matrix of the different yoghurts is. The principal

compounds were sugars and lactose, and the biggest differences between the yoghurts were in the abundance of the flavour compounds. In parallel, it was possible to verify a possible increase in the activity of some enzymes, such as acetoin reductase, diacetyl reductase, acetolactate decarboxylase and acetolactate synthase, but more studies are needed to confirm these expectations. On the other hand, β-gal, diacetyl synthase and lactate dehydrogenase possibly are not affected by pressure.

7. Organic acids and sugar content

Lactose, glucose, galactose, lactic and citric acids were identified in all samples analysed, namely at the 1st and 23rd days of storage. A chromatogram exemple is represented in **Figure II-7**, **Annex II**, and the compounds were identified by their retention time (min), namely lactose (7.39), citric acid (8.26), glucose (8.69), galactose (9.39) and lactic acid (12.91).

Lactose is the major component of milk and is the main substrate used by LAB during fermentation, producing lactic acid by glucose and galactose metabolization. It is expected that lactose decreases during fermentation and lactic acid increases, as well as lower final glucose concentration when compared with galactose concentration, since galactose is metabolized after glucose into lactic acid, as it has been previously described in the state of art. The results obtained in this analysis are in accordance with these expectations, as can be seen in **Figures 25-29**.

According to the results obtained, lactose content decrease significantly ($p < 0.05$) with pressure increase as represented in **Figure 14**, namely, comparing the control with samples fermented under 20 and 30 MPa. During yoghurt storage, between 1st and 23rd day there were no statistical differences ($p > 0.05$) in lactose content. Lactose continues to be metabolized by β-gal in glucose and galactose (reducing sugars) during cold storage.

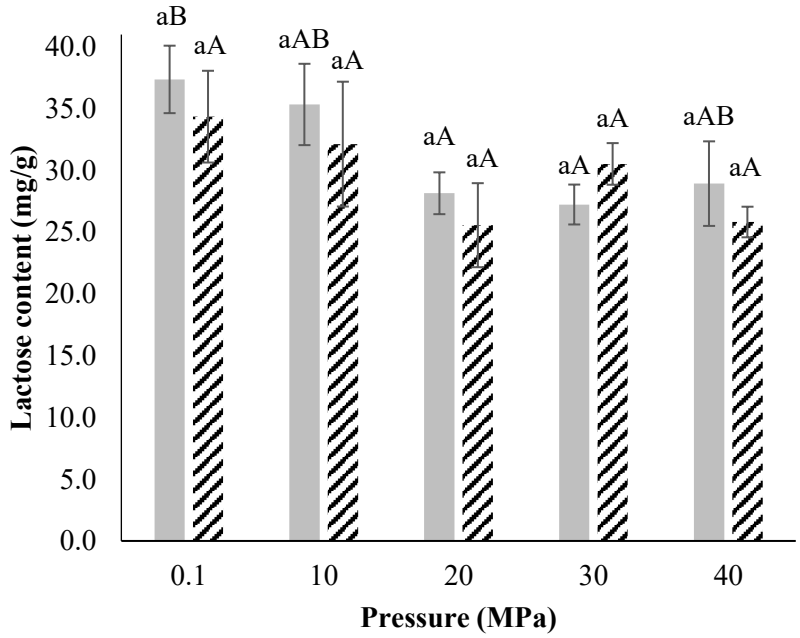


Figure 14 - Lactose content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st (■) and the 23rd (▨) day of storage. Different upper (A-B) case letters indicate statistical differences ($p < 0.05$) between and pressures during each storage day. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

In addition to lactose, galactose and glucose were also identified in the samples. During fermentation, lactose is hydrolysed by β-gal to glucose and galactose, to be transported into the cell by permeases without chemical modification. Usually, glucose is catabolized via EMP pathway, being galactose secreted from the cell (**Tamime and Robinson, 1999**). Thus, variation of galactose concentration during fermentation may be related with lactose variation, i.e. galactose concentration should increase when lactose concentration decreased.

The values obtained for glucose content are very different for the different yoghurts, as represented in **Figure 15**. The LOQ for glucose was 0.01 mg/g of yoghurt and the samples fermented under 0.1 MPa (1st and 23rd day) and 20 MPa (only for 1st day) had glucose content lower than the LOQ. Yoghurts fermented under 10 and 20 MPa had a significant increase ($p < 0.05$) of glucose during storage, which means that there was lactose metabolization by LAB during storage. However, the content in glucose did not exceed 1.5 mg/g of yoghurt for any sample. On the other hand, for yoghurts fermented under 10, 30 and 40 MPa, in the

first day of storage, some glucose was detected, which can indicate a slower fermentation rate. For yoghurts fermented under 30 and 40 MPa, glucose content variation during storage was not significant ($p > 0.05$).

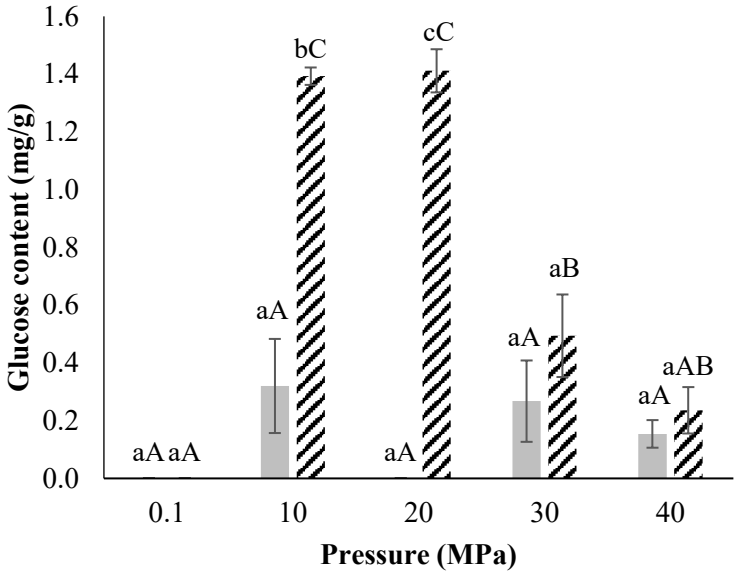


Figure 15 - Glucose content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st (■) and the 23rd (▨) day of storage. Different lower (a-c) and upper (A-C) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

In case of the other monosaccharide, galactose, its content was about 2 to 7-fold higher than glucose for the different samples and there was much higher content on the 1st day of storage, as represented in **Figure 16**. There were no significant differences ($p > 0.05$) between storage periods, except for the yoghurt samples fermented under 20 and 30 MPa, wherein an increase was observed for glucose at 20 MPa. These results show that fermentation was ongoing, and lactose continued to be metabolized as well as other minor sugars, by enzymes that can be activated by pressure. On the other hand, a bigger difference ($p < 0.05$) was observed between the yoghurts fermented under 0.1 and 10 MPa and the others, as these yoghurts had higher galactose content. This happens since galactose is not metabolized by the microorganisms of the yoghurt starter, releasing this monosaccharide into the yoghurt matrix.

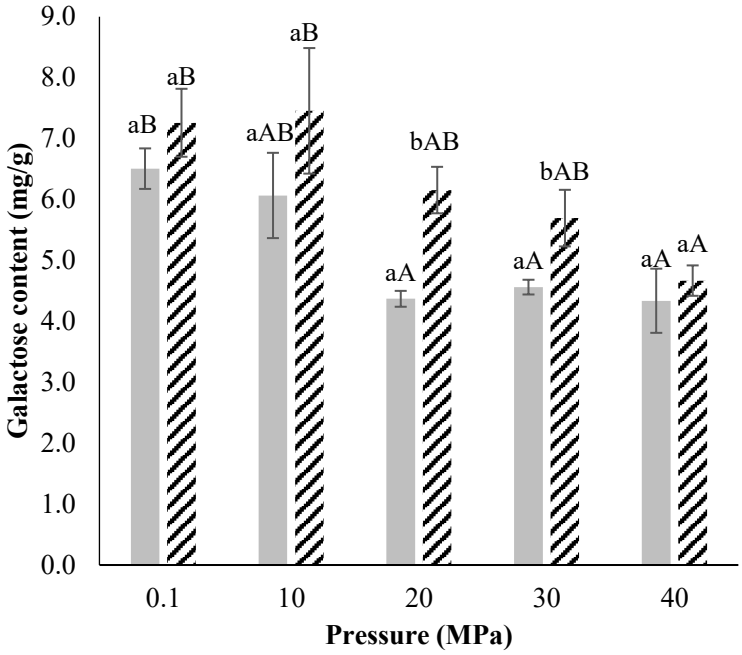


Figure 16 – Galactose content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st (■) and the 23rd (▨) day of storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Citric acid is a natural preservative present in milk, and an antioxidant. It is known that its content decreases with the age of milk (**Supplee and Bellis, 1921**), however, this content does not influence the rate of fermentation unless it is added after milk pasteurization (reduce 13.4 % of fermentation time) (**Schmidt, 2009**). In this case, the citric acid content in milk was not accessed. However, the fermentation of milk for each condition was performed in 4 consecutive days and the milk packages belonged to the same lot (batch). As such, the initial content of citric acid was expected to be similar in all milk packages. If this is correct, it means that pressure could have influenced the final content of this acid in yoghurt, as represented in **Figure 17**. In all samples, except for those fermented at 20 MPa, citric acid content did not significantly ($p > 0.05$) varied during storage. However, in all of them, except for the control sample (0.1 MPa) an increase of the average value in the 23rd day was observed. The yoghurt fermented under 20 MPa had the lower citric acid content in the first day (5.392 ± 0.172 mg/g of yoghurt) and the fermented under 0.1 MPa had the higher content

for the same day (9.134 ± 1.81 mg/g of yoghurt). These results mean that the yoghurts fermented under pressure have less citric acid content.

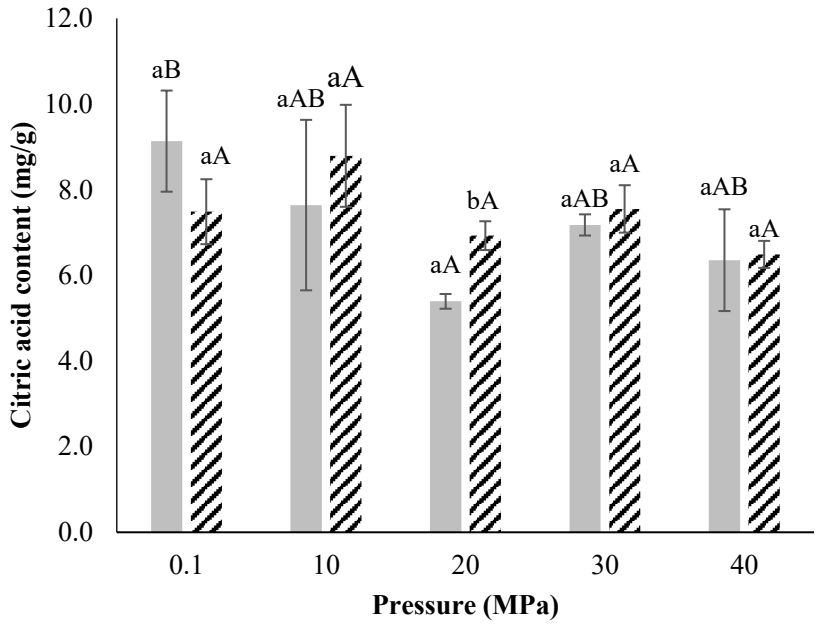


Figure 17 – Citric acid content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st (■) and the 23rd (▨) day of storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Lactic acid that is produced in the fermentation of lactose contributes to the sour taste of yoghurt by decreasing pH and grants the characteristic texture. Lactic acid content was similar to the citric acid, as represented in **Figure 18**. The yoghurt fermented under 0.1 MPa, for the 1st day of storage, presented the highest average value of lactic acid (7.893 ± 0.836 mg/g of yoghurt), however, this value is only statistically different ($p < 0.05$) from the samples fermented under 20 and 30 MPa, which had the lower content (5.209 ± 0.153 and 5.908 ± 0.051 mg/g of yoghurt, respectively). During storage there were no significant variations ($p > 0.05$), except for the yoghurt fermented under 20 and 30 MPa, for which there was an increase ($p < 0.05$) in lactic acid content was observed. These values are in accordance with the previously discussed, as lactose seems to be reduced throughout the storage. Despite of glucose and galactose increased during storage, lactic acid also increased,

which means that lactose is metabolized into glucose and galactose that contribute to the increase of lactic acid.

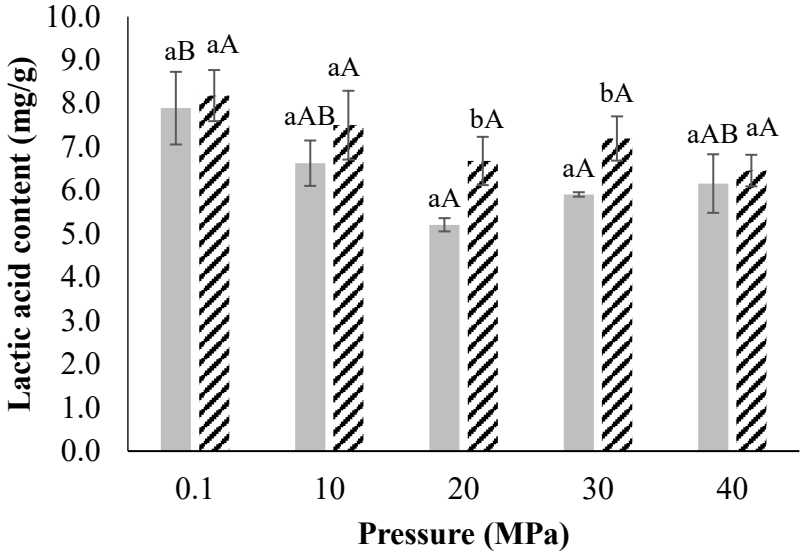


Figure 18 – Lactic acid content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st (■) and the 23rd (▨) day of storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

In general, β-gal seems to be more active when yoghurts are fermented under pressure, since lactose content at the first day of storage was lower, but more studies are needed. β-gal also remains, probably, active during storage (increase of the glucose and galactose contents) and the fermentation of lactose still slowly occurs, what can be explained by the presence of LAB and justifies the decrease of pH. The whole fresh milk used in this work had 4.8 g of sugars/ 100 mL of milk (48 mg/g), namely lactose, which means that the lactose in the control sample (yoghurt fermented under 0.1 MPa) was reduced by about 22.1 %. However, the input of pressure increases lactose metabolization: 10 MPa reduced 26.4 % of lactose, 20 MPa reduced 41.4 %, 30 MPa reduced 43.3 % and 40 MPa reduced 39.7 %.

On the other hand, the whole fresh milk used was probably rich in citric acid and is the reason why the final content in yoghurt of this acid was very similar to the lactic acid content, so, both contribute to the pH decrease. However, the samples which were fermented

under higher pressure had lower citric acid content, which suggests a catabolism of this compound during fermentation or storage, since the bacteria used cannot metabolize this acid. To sum up, the mean proportions of lactose/glucose/galactose in relation to the total sugars were similar in all yoghurts in the first day of storage, approximately: 17:0:3. However, the same did not occur on the 23rd day where the mean proportions varied with pressure (0.1, 10, 20, 30 and 40 MPa), namely 16:0:3, 15:1:4; 16:1:4; 19:0:4; 15:0:3, respectively. This means that LAB undergo different changes during fermentation and their enzymes, namely β-gal, will act differently throughout the storage. On the other hand, the mean proportions of lactose:lactate were similar in each yoghurt and in the days of storage, being about 4:1.

Lopes *et al.*, (2019) also studied the variation of sugars and organic acids in yoghurts fermented under pressure (0.1, 10 and 30 MPa) at 43ºC. In that case, the milk was reconstituted with powder milk and had 29.77 mg lactose/g. Although the initial lactose percentage was different, the results can be compared by the reduction of lactose, i.e., the intact lactose content in the yoghurt. Contrary to that observed in this work, those authors obtained a higher reduction of lactose in the control yoghurts than in the ones fermented under pressure (10 and 30 MPa), for which they obtained similar proportions of reduction. The glucose and galactose content were similar (≈1.50 and 4.00 mg/g, respectively) for all samples, which is also different from our results. The lactic acid content was similar in both works, however, in that work citric acid was not identified. These differences may be due to the different matrix and the LAB mix used.

8. TFA profile

In the fermentation process, LAB change the milk composition, such as fatty acid profiles, which can differ from one product to another. For this reason, in this work were analysed all FA, mainly the free FA and the conjugated/ esterified FA to triacylglycerols, diacylglycerols, monoacylglycerols and phospholipids to understand how different the matrix of the yoghurts fermented under pressure were.

Dietary FA components such as SFAs are associated with an increased risk of cardiovascular diseases, CHDs and mortality **(Chalabi *et al.*, 2018)**. Is recommend a limiting

SFA intake and replacing them with PUFAs and MUFAs according to some epidemiological studies and clinical trials **(Siri-Tarino *et al.*, 2010)**. **Chalabi *et al.*, (2018)** cited that dietary SFAs (C12 to C18) are indicators of atherogenic/ thrombogenic disorders whereas MUFAs, especially oleic acid, and some PUFAs such as linoleic (*n*-6) and a-linolenic acid (*n*-3), and the ratio of PUFAs to SFAs are indicators for a diet that will promote CHD. PUFAs are very susceptible to peroxidation, thereby contributing to CHDs, so, PUFA-rich diets should be consumed cautiously. Therefore, *n*-6 PUFA to *n*-3 PUFA, PUFA to SFA and MUFA to PUFA ratios could be considered as important parameters by which to determine the nutritional value of a food (**Butler *et al.*, 2011**). The aim of this FA study is to compare the FA composition and related lipid quality of yoghurt fermented under pressure and the conventional one (fermented under 0.1 MPa).

According to the number of carbon atoms and dietary safety, the identified FA were divided into three main groups: short-chain FA (SCFAs) (C4, C6, C8 and C10), SFAs (C12, C14, C15, C16, C17, C18, C20, C22 and C24), and USFAs including MUFAs (C10:1 t2, C12:1, C14:1 c9, C15:1, C16:1 c7, C16:1 c9, C17:1 c10, C18:1 t12, C18:1 c9, C18:1 t15 and C18:1 c11) and PUFAs (C18:2 c9, c12 (*n*-6), C18:3 c9, c12, c15 (*n*-3), C18:9 c9, t11 (CLA) and C20:4 c5, c8, c11, c14). Moreover, there were identified some isomers (i) and anti-isomers (ai) of some FA (C13i, C13ai, C14i, C17i, C17ai). A chromatogram example is represented in **Figure II-8 (Annex II)** and the compounds were identified by their retention time (**Table 16**) comparing with other yoghurt spectra.

Table 16 – Fatty acids (FA) identified group profile during storage expressed in percentage (%) of each yoghurt fermented under different pressures (0.1, 10, 20, 30 and 40 MPa) at 43ºC (n=3).

Lipid numbers	Common name	Systematic name	Retention time (min)
C4	Butyric acid	Butanoic acid	3.80
C6	Caproic acid	Hexanoic acid	7.10
C8	Caprylic acid	Octanoic acid	10.0
C10	Capric acid	Decanoic acid	12.2
C10:1 (t2)	Decenoic acid	trans-2-decenoic Acid	12.7
C12	Lauric acid	Dodecanoic acid	13.9
C13 i	Tridecylic acid (isomer)	Tridecanoic acid	14.3
C13 ai	Tridecylic acid (anti-isomer)	Tridecanoic acid	14.5
C12:1	Lauroleic acid	9-dodecenoic acid	14.6
C14 i	Myristic acid (isomer)	Tetradecanoic acid	15.5
C14	Myristic acid	Tetradecanoic acid	16.0
C14:1 (c9)	Myristoleic acid	cis-9-tetradecenoic acid	16.7

C15	Pentadecylic acid	Pentadecanoic acid	16.9
C15:1	Pentadecenoic acid	14-pentadecenoic acid	17.3
C16	Palmitic acid	Hexadecanoic acid	18.8
C16:1 (c7)	Palmitoleic acid	cis-7-hexadecanoic acid	19.4
C16:1 (c9)	Hexadecenoic acid	cis-9-hexadecanoic acid	19.5
C17 i	Margaric acid (isomer)	Heptadecanoic acid	19.7
C17 ai	Margaric acid (anti-isomer)	Heptadecanoic acid	20.0
C17	Margaric acid	Heptadecanoic acid	20.5
C17:1 (c10)	Heptadecenoic acid	cis-10-heptadecenoic acid	21.3
C18	Stearic acid	Octadecanoic acid	22.5
C18:1 (t12)		trans-12-octadecanoic acid	23.1
C18:1 (c9)	Oleic acid	cis-9-octadecanoic acid	23.3
C18:1 (t15)		trans-15-octadecanoic acid	23.5
C18:1 (c11)	Vaccenic acid	cis-11-octadecanoic acid	23.6
C18:2 (c9,c12)	Linoleic acid	all cis-9,12-octadecanoic acid	24.7
C18:3 (c9,c12,c15)	α-Linolenic acid	all cis-9,12,15-octadecanoic acid	26.5
C20	Arachidic acid	Eicosanoic acid	27.0
CLA - C18:2 (c9,t11)	Conjugated linoleic acid (Rumenic acid)	cis-9-trans-11-octadecanoic acid	27.8
C20:4 (c5,c8,c11,c14)	Arachidonic acid	all cis-5,8,11,14-eicosanoic acid	30.6
C22	Behenic acid	Docosanoic acid	37.3
C24	Lignoceric acid	Tetracosanoic acid	37.1

In all samples it was possible to identify and quantify 33 FA, whose content was higher than the LOQ. Our results showed that the FA profiles and their content of a sample fermented under each pressure does not change significantly ($p > 0.05$) along refrigerated storage. However, the yoghurts fermented under different pressures had different FA content in the both storage days studied, as seen in **Tables II-(3-4)** present in **Annex II**.

The milk used had 3.6 g of fat/100 mL of milk and 2.4 g of that are SFA (**Table I-1, Annex I**). In terms of TFA, the yoghurt fermented under atmospheric pressure presented higher content 28006.5 ± 2547.1 μg/mg of yoghurt (1st day of storage) and with the increase of the applied pressure the content in TFA decrease 5.4, 14.6, 53.0 and 56.1 % for yoghurts fermented under 10, 20, 30 and 40 MPa respectively. This decrease is also noted in some groups of FA (SCFA, SFA and MUFA) and the more noticeable differences are between the yoghurts fermented under low pressures (0.1 and 10 MPa) and the fermented under higher pressures (20, 30 and 40 MPa) ($p < 0.05$) as seen in **Annex II - Table II-3**. These results suggest that FA might be being used by LAB (to take energy or to adapt their membrane to

assure pressure resistance, as it will be explained below) or being led to the formation of volatile compounds. The most interesting case is the yoghurt fermented under 10 MPa that had higher content in PUFA but also in *n*-3 and *n*-6 FA for the first day of storage.

The relative content of FA in yoghurts are represented in **Table 17** and expressed as percentages. Although TFA content decreases, the relative percentage of each FA groups does not maintain relative to its TFA content. This means that each fatty acid may be affected differently (by increasing or decreasing their content) when the fermentation pressure increased. Indeed, it appears that by increasing the fermentation pressure, the relative percentage of total saturated fatty acids (SCFA + SFA) is higher in fermented yoghurts under pressure, however for MUFA and total FA *n*-6 tend to decrease with the pressure increase. The relative percentage of PUFA, total FA *n*-3 and trans-FA are higher in yoghurts fermented under 10, 20 and 30 MPa.

Table 17 – Changes in fatty acid (FA) group profile along storage, expressed in percentage (%) of each yoghurt fermented under different pressures (0.1, 10, 20, 30 and 40 MPa) (n=3).

	Pressure (MPa)	**0.1**		**10**		**20**		**30**		**40**	
	Day of storage	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**
Fatty acids (%)	**SCFA +SFA**	15.6	16.0	16.1	16.6	16.3	16.8	17.2	17.0	17.6	17.2
	MUFA	80.9	80.4	79.9	79.3	79.6	79.2	78.7	78.8	78.8	79.2
	PUFA	3.5	3.5	4.1	4.2	4.1	4.0	4.0	4.1	3.6	3.7
	Total FA n-3	2.1	2.2	2.6	2.7	2.7	2.6	2.6	2.7	2.2	2.2
	Total FA n-6	0.7	0.6	0.7	0.6	0.6	0.4	0.3	0.4	0.3	0.3
	FA cis	50.6	50.5	51.2	51.0	51.1	50.8	50.4	50.6	49.6	49.7
	FA trans	32.2	31.9	31.2	30.8	31.0	30.8	30.8	30.8	31.2	31.4

Note: Short-chain fatty acids and short fatty acids (SCFA+ SFA) monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); Total fatty acids omega 3 (Total FA *n*-3); Total fatty acids omega 6 (Total FA *n*-6); Total cis unsaturated fatty acid (FA *cis*); Total trans unsaturated fatty acid (FA *trans*);

As the author is aware, this is the first time that a yoghurt fermented under pressure is characterized according to its FA profile, however the FA content of the yoghurt fermented at atmospheric pressure is according with some authors (**Chalabi *et al.*, 2018; Güler and Gürsoy-Balcı, 2011; Júnior *et al.*, 2012**). However, there are others studies concerning the effects of high pressure on fatty acids, however, just were noted changes when are applied higher pressures in meat (> 350 MPa, during 20 min at 20°C) (**He *et al.*, 2012**), other study concluded that pressure (700 MPa) induces some conformational changes at the hydrocarbon skeleton on USFA in solid samples, while the liquid ones remain unchanged (**Povedano *et***

***al.*, 2014**), even though the results cannot be compared, as this work aimed a different range of pressures (10–40 MPa) during a long period of time at higher temperatures (43°C).

The membrane of LAB can be modified due to pressure applied and to perform physiological functions in hostile environments, bacteria potentially remodel the membrane by changing the ratio of **(i)** saturation to unsaturation, **(ii)** *cis* to *trans* unsaturation, **(iii)** branched to unbranched structure, and **(iv)** acyl chain length. FA containing single or more unsaturated bonds have more bulky conformation than their saturated counterparts do, thus allowing higher conformational freedom and lesser packing of the membrane (**Abe, 2015**).

Natural cell membranes are a complex mixture of phospholipids, sterols, and numerous membrane proteins. Therefore, it is difficult to provide a straightforward account of the effect of high pressure on the phase behaviour of the membranes, their structure, activity of membrane proteins, and cell growth and viability (**Abe, 2015**). However, **Beal *et al.*, (2001)** studied the FA composition of the cell membrane of *S. thermophilus* and their change by alteration of some factors: incorporating oleic acid in the culture medium, fermentation pH, addition of glycerol as cryoprotective agent and duration of storage (at -20ºC). Firstly, there were identified nine FA in the cell membrane of *S. thermophilus*, namely C14:0, C16:0, C16:1, C18:1 c9, C18:1 c11, C19:1, C20:0, C20:1, the same that were identified by other authors in *L. bulgaricus* except C20:1. When the culture media was incorporated with oleic acid (C18:1 c9), the content in SFA decrease (C14:0, C16:0, C18:0, C20:0) but the content in C18:1 c9, C19:1 and C20:1 increase, so the ratio of USFA/SFA increased. The same was noticed when the fermentation pH decreased to 5.5. These results suggest that FA incorporated in milk must be integrated into LAB cell membrane due to the content in oleic acid, pH diminishing and pressure, although there is no evidence of this latter factor. Our results are in accordance with the results obtained by these authors, as represented in **Table 17** the MUFA content (% of TFA) decrease, mainly C18:1 c9 (**Annex II - Table II-1**), and the SFA content increase (% of TFA) that means that the SFA of LAB cell membrane are replaced by MUFA to increase their pressure resistance.

Nevertheless, it is known the importance of fat in the perception of food, and to modify the physical properties of food, including mouthfeel, appearance and structure. Fat is also important as a flavour precursor, flavour carrier and flavour release modulator, for these reasons it is very important a volatile compounds study to understand if these FA are possibility used as its precursors.

In parallel, we also studied some parameters/index to understand the nutritional quality of each yoghurt. IA, IT and the ratio of omega-6/omega-3, MUFA/PUFA, and the PUFA/SFA were calculated (**Table** 18). IA and IT index were very similar for all yoghurts along the storage. It is perceptible that both atherogenic and thrombogenic indices are very low, which can be attributed to the higher content in USFA comparing to C12, C14, C16 and C18. Note that C14, C16 and C18 are associated with high serum cholesterol and low density lipoprotein (LDL) cholesterol levels as risk factors for CHD and C18 is a thrombogenic SFAs, which accelerates blood clotting and the formation of platelet aggregation (**Briggs et al., 2017; Müller et al., 2003**).

Moreover, these results revealed that the *n*-6/*n*-3 and MUFA/PUFA ratios are higher for yoghurts fermented under 0.1 MPa and lower for the fermented under 20 and 30 MPa, that is probably a good result, as FA *n*-3 should prevail, because all intermediates of lipid metabolism from linoleic acid (*n*-6) are more harmful, for example prostaglandins and leukotrienes that are thrombogenic agents. Also, an excessive intake of PUFAs exerts undesirable effects such as oxidative stress induction and the *n*-6/*n*-3 ratio (as an index) is used in the prognosis of heart disease, diabetes and obesity, and many studies recommend that this ratio should be below 4 - a ratio of 4/1 was associated with a 70% decrease in total mortality (**Simopoulos, 2016, 2008**). On the other hand, the MUFAs are as effective as PUFAs in lowering serum cholesterol and the MUFA/PUFA ratio of a diet can be used as an indicator for protection from heart diseases (**Naydenova *et al.*, 2014**). On the other hand, our findings indicate that the PUFA/SFA ratios in the control and for all samples fermented under pressure were lower than 0.4, which is in accordance with the recommendations made by the World Health Organization (WHO) (**Expert Consultation on Diet, Nutrition, and the Prevention of Chronic Diseases *et al.*, 2003**).

Table 18 - Lipid quality indices of yoghurt fermented under 0.1, 10, 20, 30 and 40 MPa for the 1st and 23rd day of storage (n=3).

Quality parameter	IA		IT		*n*-6/*n*-3		MUFA/PUFA		PUFA/ (SCFA+SFA)	
Pressure (MPa)	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**
0.1	0.06	0.06	0.03	0.03	0.32	0.29	23.04	22.71	0.23	0.22
10	0.06	0.06	0.03	0.03	0.28	0.26	19.69	19.08	0.25	0.25
20	0.06	0.06	0.03	0.03	0.25	0.24	19.51	19.70	0.25	0.24
30	0.06	0.06	0.03	0.03	0.25	0.26	19.47	19.13	0.23	0.24
40	0.06	0.06	0.03	0.03	0.29	0.28	21.86	21.66	0.21	0.21

Index of atherogenicity (IA); index of thrombogenicity (IT); Omega-6/omega-3 (*n*-6/*n*-3); monounsaturated/polyunsaturated fatty acid (MUFA/PUFA); polyunsaturated/short-chain fatty acids and saturated fatty acid (PUFA/SFA);

9. Volatile compounds profile

Volatile compounds from fermented milk products are very diverse and sometimes make a difficult and delayed identification. The minor and major compounds were identified in all samples for yoghurts fermented under 0.1, 10, 20, 30 and 40 MPa, for the 1st and 23rd day and are described in **Table 19**, divided in groups of acids, aldehydes, ketones, alcohols, esters, ethers, aromatic, heterocyclic, terpenes, sulphur and carbonyl compounds. The criterion used for exclusion or choice the more probable compounds was based on the relative match equal to or greater than 700 (R. match ≥ 700), resulting 131 volatile compounds identified, more than the described in **Table 6** (but some of the identified compounds are different in the both tables). The principal compounds described in the literature, mainly acetaldehyde, acetone, acetoin and diacetyl were founded in all samples, however it was only possible to identify a derivate of 2-butanone (2-Butanone, 3,4-epoxy-3-ethyl-).

It is important to refer that the yoghurts are heated at 60°C to do a extraction of the volatile compounds by SPME and probably some of the identified compounds are resulted of chemical reactions by the increasing of temperature, so it is possible that some compounds are not present in the fresh yoghurt.

Moreover, by chromatograms observation (**Figure II-9, Annex II**), probably the major compounds decrease their content along the storage (less content in the 23rd day of storage) as some authors verified for other yoghurts fermented under 0.1 MPa **(Dan *et al.*, 2017)**. On the other hand, it seems like higher pressure decrease the content of some compounds but

causes the appearance of others, as described in **Table 19** (qualitative results). Nevertheless, to confirm these conclusions, it is needed to do a semi-quantitative analysis for all identified compounds.

Table 19 - Volatile compounds identification by their retention time (RT) (min) with relative match equal or greater than 700 (R.match $\geq$ 700). The evaluation qualitative was done by their identification/ presence in some samples. The analysis were done in triplicate.

Group	Compound	RT (min)	Presence		
			in all samples	except in	only in
Acids	Tetradecanoic acid	36.11	X		
	Acetic acid	39.391; 39.781	X		
	2-Nonenoic acid	42.55	X		
	Acetic acid, N'-[3-(1-hydroxy-1-phenylethyl)phenyl]hydrazide	43.26	X		
	Formic acid	44.23		20, 30 and 40 MPa	
	Propanoic acid, 2-methyl-	46.14	X		
	Butanoic acid	49.63	X		
	4-Methyloctanoic acid	50.06	X		
	Hexanoic acid, 2-methyl-	51.93	X		
	Pentanoic acid	55.74	X		
	Hexanoic acid	61.45	X		
	Hexanoic acid, 2-ethyl-	66.54	X		
	Heptanoic acid	66.78	X		
	Octanoic acid	71.96	X		
	Nonanoic acid	76.82	X		
	n-Decanoic acid	81.5	X		
	9-Decenoic acid	84.13	X		
	Undecanoic acid	85.97	X		
	Benzoic acid	88.25	X		
	Dodecanoic acid	90.28	X		
	Tetradecanoic acid	98.35	X		
	n-Hexadecanoic acid	105.9	X		

Alcohols	1,3-Butanediol, (S)-	6.655	X		
	1-hexanol-2-ethyl	16.8			10 MPa (23rd day)
	1-Pentanol	22.15			30 and 40 MPa
	4-Ethylcyclohexanol	23.81			30 and 40 MPa
	1-Pentanol	25.36		40 MPa	
	2-Hexanol, 3-methyl-	28.2	X		
	3-Pentanol, 2-methyl-	31.26	X		
	1-Hexanol	32.35	X		
	1-Pentanol, 3-methyl-	32.43	X		
	2-Pentanol, 3-methyl-	32.45	X		
	2-Hexanol	32.57	X		
	4-Methyl-2-hexanol	32.59	X		
	2-Nonen-1-ol, (Z)-	34.5	X		
	2-Nonen-1-ol, (E)-	34.61	X		
	2-Octen-1-ol	34.67	X		
	1-Octen-3-ol	38.69	X		
	1-Heptanol	38.98	X		
	5-Methyl-1-hepten-4-ol	39.59			30 and 40 MPa
	1,7-Octanediol, 3,7-dimethyl-	39.96	X		
	trans-2-Ethyl-2-hexen-1-ol	41.58	X		
	3-Hexyne-2,5-diol	42	X		
	1-Hexanol, 3,5,5-trimethyl-	42.12	X		
	Linalool	44.72	X		
	1-Octanol	45.28	X		
	2-Methyl-5-hexen-3-ol	46.1	X		
	2-Octen-1-ol, (E)-	48.71	X		
	Ethanol, 2-(2-ethoxyethoxy)-	48.81	X		
	1-Nonanol	51.23	X		
	L-α-Terpineol	53.02	X		
	2-Nonen-1-ol	54.28		0.1 and 10 MPa	
	1-Decanol	56.89	X		
	5-Hexyn-1-ol	58.9	X		
	Phenol	69.41	X		
	1-Hexanol, 2-ethyl-	41.17	X		
	trans-2-Ethyl-2-hexen-1-ol	48.56	X		

Aldehydes	Acetaldehyde	4.156	X		
	Hexanal	13.01	X		
	Heptanal	20.07	X		
	2-Butenal, 3-methyl-	21.4	X		
	2-Heptenal, (E)-	22.55	X		
	2-Hexenal, (E)-	22.76		30 and 40 MPa	
	Octanal	27.28	X		
	2-Heptenal, (Z)-	29.74	X		
	2-Heptenal, (E)-	29.76	X		
	Nonanal	34.52	X		
	2-Octenal, (E)-	36.78	X		
	2-Octenal, (E)-	36.82	X		
	2-Nonenal, (E)-	43.49	X		
	Undecanal	54.29			40 MPa
	2-Undecenal, (E)-	55.95	X		
	Benzaldehyde	42.84	X		
	2,5-Dihydroxybenzaldehyde, 2TMS derivative	45.55	X		
Aromatic	Benzeneethanamine	80.59			30 and 40 MPa
	Benzene, (1-methylethyl)-	22.07	X		
Esters	Oxalic acid, 2-ethylhexyl nonyl ester	11.51	X		
	Acetic acid, hexyl ester	26.25	X		
	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester	62.19		40 MPa	
	Pentanoic acid, 2,2,4-trimethyl-3-hydroxy-, isobutyl ester	62.68	X		
	Methoxyacetic acid, tetradecyl ester	67.93	X		
	Ethanol, 2-nitro-, propionate (ester)	44.54	X		
	di-tert-Butyl dicarbonate	12.94	X		
	δ-Dodecalactone	87.49	X		
	Ethyl 4-hydroxymandelate, 2TMS derivative	45.81	X		
	Tetraacetyl-d-xylonic nitrile	16.8			0.1 MPa
	Triacetin	72.98		30 and 40 MPa	
Ethers	Ether, 6-methylheptyl vinyl	21.34			0.1 MPa
	Ether, 6-methylheptyl vinyl	45.38	X		

Heterocyclic	Furan, 2-pentyl-	22.74	X		
Carbonyl	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	9.346	X		
	Octane, 1,1'-oxybis	50.92	X		
	Cyclobutene, 2-propenylidene-	11.03	X		
	Undecane	14.03	X		
	5-Undecene, 3-methyl-, (Z)-	17.05			30 and 40 MPa
	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-	19.33		30 MPa	
	Cyclohexene, 4-ethenyl-1,4-dimethyl-	19.51		30 MPa	
	Tetradecane	20.7		30 MPa	
	Undecane	20.85		30 MPa	
	Dodecane	21.22		0.1 MPa	
	Tetradecane	21.5			20 MPa - 23rd day; 30 MPa - 1st day; 40MPa
	Hexane, 3,3,4,4-tetrafluoro-	22.08		20 and 30 MPa	
	7-Oxabicyclo[4.1.0]heptane	22.35		30 MPa	
	o-Cymene or p-Cymene	25.6			30 and 40 MPa
	Octane, 4-chloro-	29.69	X		
	Ethene, fluoro-	44.38		30 and 40 MPa	
	Cyclopentane	58.14	X		
	Silane, trichlorodocosyl-	45.42	X		
	2,4-Octadiyne	16.16			40 MPa
	Cyclopentene, 1-butyl-	35.75			30 and 40 MPa
Ketones	Acetone	4.74	X		
	2,3-Butanedione (Diacetyl)	7.961	X		
	Cyclohexanone, 4-hydroxy-4-methyl-	47.11	X		
	2-Undecanone	47.41	X		
	2-Tridecanone	47.6	X		
	Acetophenone	50.35	X		
	2-Tridecanone	59.17	X		

	2-Undecanone, 6,10-dimethyl-	59.17	X		
	Dimethyl sulfone	64.44	X		
	2H-Pyran-2-one, tetrahydro-6-propyl-	67.01	X		
	2H-Pyran-2-one, tetrahydro-6-pentyl-	77.62	X		
	2,3-Pentanedione	12.31	X		
	2-Heptanone	19.6	X		
	2-Octanone	20.01	X		
	Cyclopentanone, 2-methyl-	26.87	X		
	Cyclohexanone (internal standard)	26.91	X		
	1-Octen-3-one	28.26	X		
	2-Butanone, 3,4-epoxy-3-ethyl-	30.25	X		
	5-Hepten-2-one, 6-methyl-	30.89	X		
	2-Hydroxy-3-pentanone	32.45	X		
	2-Hydroxy-3-pentanone	32.49	X		
	Dimethyl trisulfide	33.06			30 and 40 MPa
	2-Nonanone	34.18	X		
	[1,1'-Bicyclopentyl]-2-one	53.51	X		
	Acetoin	27.54	X		
	8-Nonen-2-one	37.93		0.1 and 10 MPa	
	2-Nonen-4-one	40.17	X		
	2-Decanone	40.96		0.1 and 10 MPa	
Sulphur	Methanesulfonyl chloride	22.02	X		
Terpenes	β-Pinene	13.423; 13.521	X		
	β-Myrcene	13.46	X		
	α-Pinene	9.19	X		
	β-Myrcene	18.09			40 MPa
	p-Xylene	19.1	X		
	D-limonene	19.27		20 and 30 MPa	

In the cases where the "presence" are only described as the fermentation pressure, means that the compound were observed in the two days of analysis (1st and 23rd days). When differences are only observed in one day, this day is described in the table.

10. Sensorial analyses

Eight (8) usual yoghurt consumers within our research group (master and PhD students) evaluated five (5) different yoghurts (produced at 0.1, 10, 20, 30, and 40 MPa), whose fermentation conditions were unknown (identified with a letter code) (**Figure 19**). Yoghurt fermented at 10 MPa exhibited the highest preference (50 %). The yoghurts fermented at 0.1 and 20 MPa presented the same percentage of preference (25 % each one). In terms of flavour, yoghurt fermented under 10 MPa had the best score, followed by the fermented under 0.1 MPa and 20 MPa (38, 37 and 25 %, respectively). In terms of texture, the consumers prefer the one that was fermented at 10 MPa (63 %) than the yoghurt fermented at atmospheric pressure (37 %). On the other side, yoghurts fermented at 30 or 40 MPa were less appreciated by the panel in any of the parameters and a cheese-like odour was reported. So, the yoghurt fermented at atmospheric pressure has a stronger smell and taste than those fermented at 10 and 20 MPa. However, yoghurt fermented at 40 MPa seems to have a cheese-like flavour. Despite the low number of participants in the sensory analysis, it is important to highlight the higher acceptability and preference of the yoghurt fermented under 10 and 20 MPa, particularly the former.

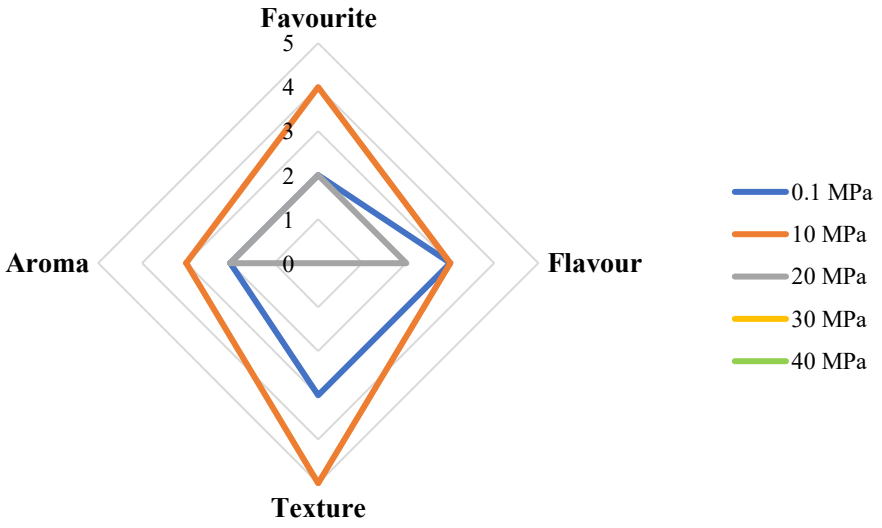


Figure 19 - Yoghurt sensorial analysis by a non-trained panel of 8 yoghurt consumers. Yoghurts fermented under 30 and 40 MPa were not selected as preferred for any of the categories evaluated and so these results are not visible in the graph.

CHAPTER IV – CONCLUSIONS

This was the first study about yoghurt (fermented under pressure) conservation for 23 days and, in addition to the results reported in the literature concerning yoghurt fermentation under pressure, it was possible to observe interesting results. The fermentation time to produce yoghurt increases directly with the fermentation pressure, with a similar final pH of ≈4.5 for all samples, until 40 MPa. Moreover, yoghurts fermented at 0.1, 10, 20 and 30 MPa presented significantly higher microbial counts than those fermented at 40 MPa, but this difference was less evident along storage. Despite some variations of the pH throughout storage, the initial (1st day) and final (23rd day) pH was similar for all yoghurts. Relatively to TA and colour parameters, pressure did not affect them, but increased syneresis. In general, pressure seems to increase the firmness of yoghurt but decrease plastic adhesiveness.

Despite of the microbiological, physicochemical and texture study, the yoghurts fermented were characterized for better LAB metabolism understanding. With the metabolomics assessment by NMR, it was noted that the compounds 2,3-butanediol, acetoin, diacetyl and formate vary with pressure increase and it was speculated that some enzymes are affected by pressure: the activity of diacetyl reductase, acetoin reductase and acetolactate decarboxylase may increase, while β-gal, diacetyl synthase and lactate dehydrogenase seem not to be affected (positively) by pressure. These results need to be confirmed by further and deeper studies concerning the enzymes affected by lower/medium pressures.

In terms of consumed sugars, the pressure increase led to higher lactose consumption, while the control samples (yoghurt fermented at 0.1 MPa) were reduce about 22.1 % of lactose but the one's fermented at 40 MPa were reduced in 39.7 %. On the other hand, the mean proportions of lactose/ lactic acid were similar for each yoghurt and for the days of storage, been about 4:1, this means that yoghurts fermented under pressure converts in the same proportion lactose to lactic acid when compared with the fermented under atmospheric pressure. The mean proportions of lactose/glucose/galactose, in relation to the total sugars, vary during storage, and for yoghurts fermented under 10, 30 and 40 MPa in the first day of storage it was observed some glucose content, that can indicate a more slower fermentation, as it was verified by fermentation time, but along the storage its content increases, suggesting that LAB (when not submitted to the hydrostatic pressure stress) increases the fermentation rate, as it was verified as pH decrease along refrigerated

storage. The galactose content is much higher than the content in glucose, but it decreases with pressure increase, however, it does not change significatively during storage. These results mean that galactose is not totally metabolized by the LAB, releasing this monosaccharide into the yoghurt matrix.

The TFA content were also studied and seems to decrease with pressure increase, but also, the relative percentage of each FA groups does not maintain. With the increase of pressure, the relative percentage of total saturated FA (SCFA + SFA) is higher in fermented yoghurts under pressure, however for MUFA and total FA *n-6* tend to decrease with the pressure increase. The relative percentage of PUFA, total FA *n-3* and FA trans was higher in yoghurts fermented under 10, 20 and 30 MPa. Possibly the SFA of LAB cell membrane are replaced by MUFA to increase their pressure resistance, which may be a possible explanation for the results obtained, however, FA also can be used by LABs to take energy or to produce more volatile compounds. The consuming of these yoghurts can be a good consumer choice because have less sugar and fat content, but also the lipid quality indices obtained were very good and contribute to healthy diet.

The method applied to determine volatile compounds allowed identifying 131 compounds, but many of them only appear in the yoghurts fermented under higher pressures. The principal compounds described by literature, mainly acetaldehyde, acetone, acetoin and diacetyl were present in all samples and along the storage. Moreover, by chromatograms observation, probably the major compounds decrease their content along the storage and, on the other hand, it seems like higher pressure decrease the content of some compounds but causes the appearance of others but to confirm these conclusions and the metabolomics (by NMR) conclusions about these compounds, it is needed to do a semi-quantitative analysis for all the identified compounds.

Finally, and despite of the low number of participants in the sensory analysis, it is important to highlight the higher acceptability and preference of the yoghurt fermented under pressure, mainly at 10 and 20 MPa. The yoghurts fermented under 30 and 40 MPa were the less appreciated, and probably due to the presence of the different identified volatile compounds.

Although the yoghurt fermentation under pressure is slower than the conventional and the energy required to produce them can be slightly higher, it seems that these yoghurts have potential and appear to be healthier, possibly, than the conventional fermented at

atmospheric pressure. However, further studies are of interest to better understand the behaviour of LAB under pressure and possible health benefits.

CHAPTER V – FUTURE WORK

Evidences show that HPP may have interesting applications to modify milk structure and composition for fermented products production, as shown by the results described in this thesis. However, it is important understand more about the LAB behaviour under pressure and how do they tend to respond to the pressure sub-lethal stress. More and deeper analyses are needed to understand how the LAB metabolism behaves when low pressures are applied during a long time. The study of LAB cell membrane change is an important parameter to confirm some expectations, the proteolysis assessment, the antioxidant capacity and digestibility assessment to understand if that yoghurts fermented under pressure are more digestible and beneficial than the traditional counterpart, but also, angiotensin-I-converting enzymes-inhibitory activity assessment to secure possible advantages in blood pressure control.

Besides milk processing, pre-treatment of starter LAB may also enhance fermented dairy products' quality/bioactivity, yet there are no studies on the use of HPP, PEF or US for such pre-treatment. Moreover, there is no information about these last technologies, PEF and US, when are applied alone or in combination in lactic acid bacteria or lactic yeasts. So, the study of dairy products under different and combined sub-lethal conditions/ factors it is an opportunity to contribute to scientific research, increasing the knowledge of microorganism's metabolism, and also for industrial improvement if fermentation rate can be increased. Furthermore, it is possible to obtain new/different dairy products, with potential to be beneficial to consumers' health.

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CHAPTER VII – ANNEXS

Annex I

Materials and Methods

Table I-1 - *Vigor* whole milk nutritional table.

Composition (unit)	***Vigor* (Whole) /100 mL**
Energy (KJ/Kcal)	271/65
Lipids (g)	3.6
Saturated (g)	2.4
Carbon hydrates (g)	4.8
Sugars (g)	4.8
Proteins(g)	3.3
Salt → sodium (g)	0.10
Vitamins and minerals	
Calcio (mg)	120
Vitamin B12 (µg)	0.40
Vitamin A (µg)	0
Vitamin D (µg)	0
Vitamin E (mg)	0

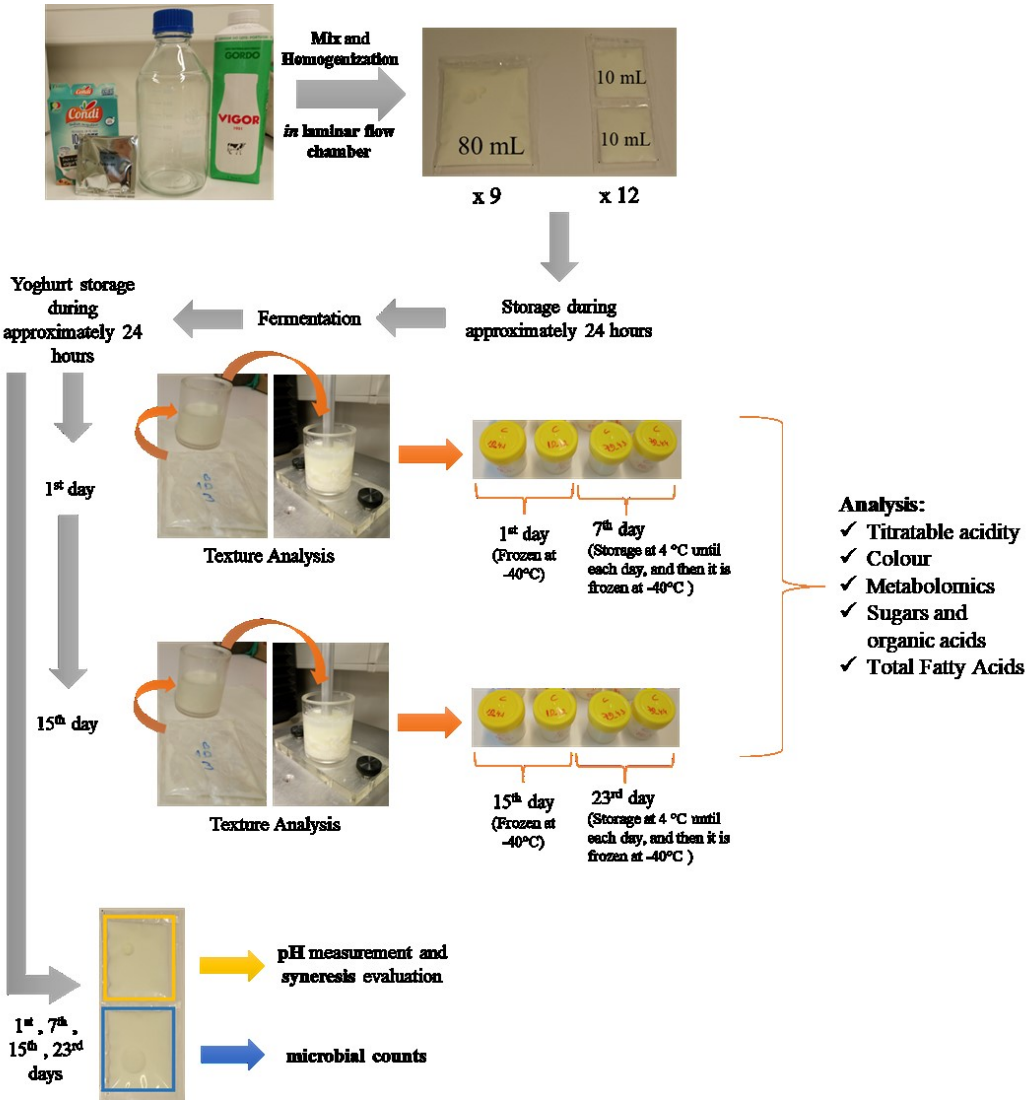


Figure I-1 - Sample preparation and respective analysis flow chart.

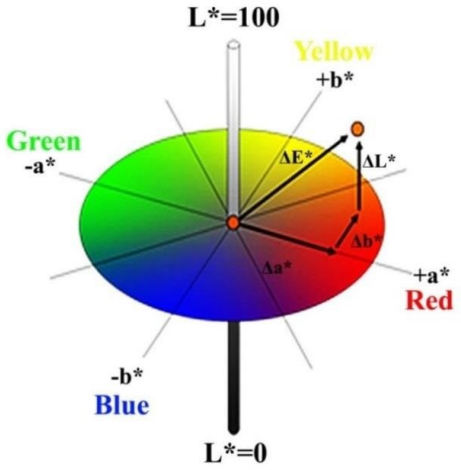
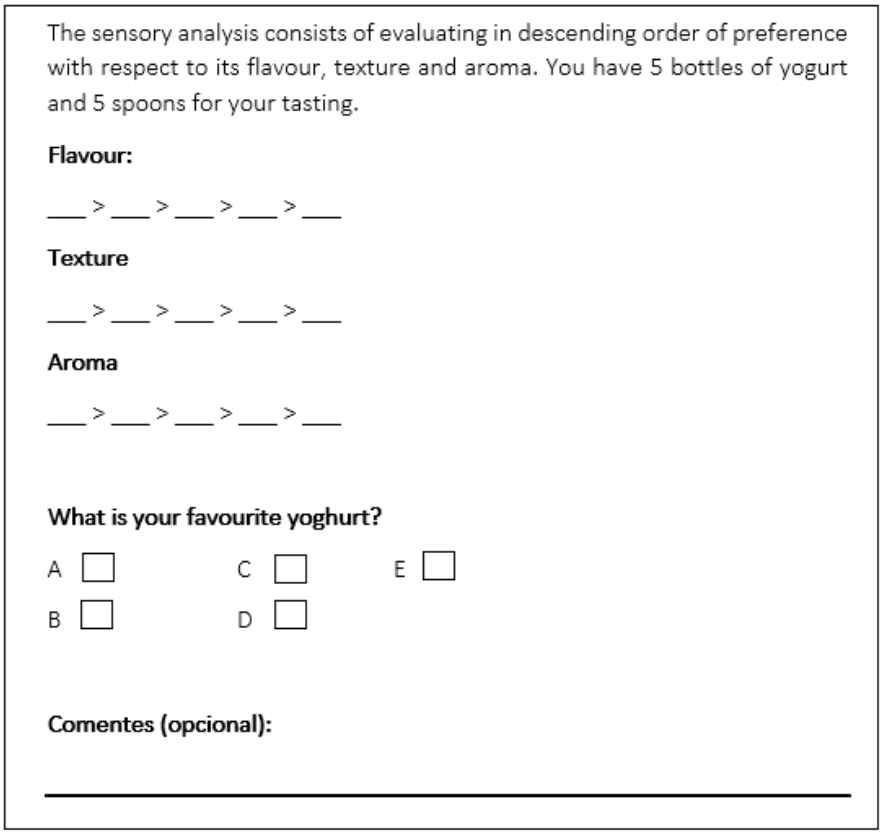


Figure I-2 - Colour measurement according different parameters.


The sensory analysis consists of evaluating in descending order of preference with respect to its flavour, texture and aroma. You have 5 bottles of yogurt and 5 spoons for your tasting.

Flavour:

___ > ___ > ___ > ___ > ___

Texture

___ > ___ > ___ > ___ > ___

Aroma

___ > ___ > ___ > ___ > ___

What is your favourite yoghurt?

A ☐ C ☐ E ☐

B ☐ D ☐

Comentes (opcional):

Figure I-3 - Document for sensory analysis to obtain the anonymous consumer opinion.

Annex II

Results and Discussion – Complementary results

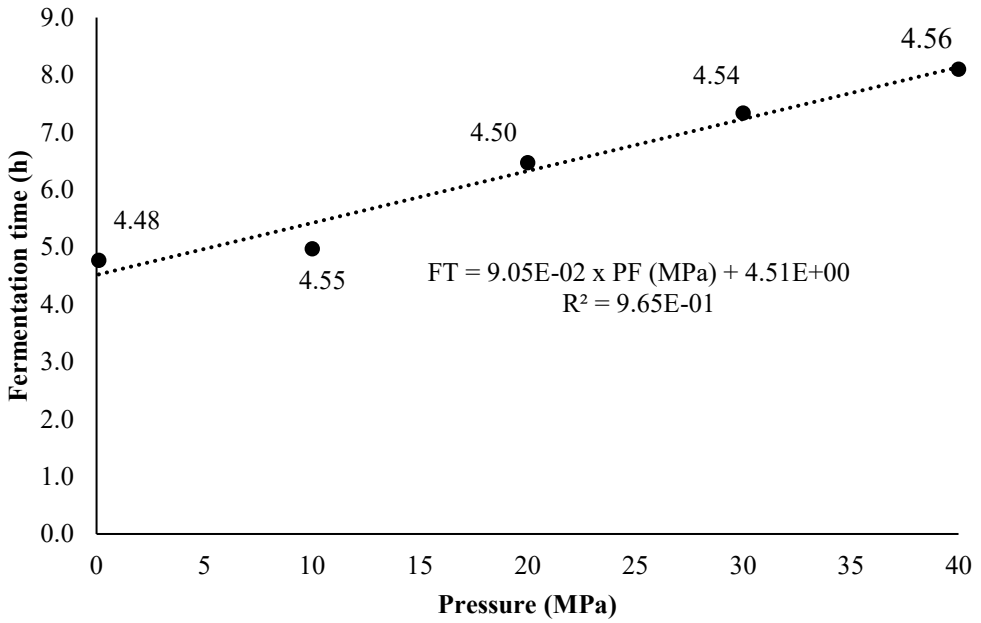


Figure II-1 - Variation of fermentation time (h) according to the set fermentation pressure (MPa). Near to each point is represented the final pH.

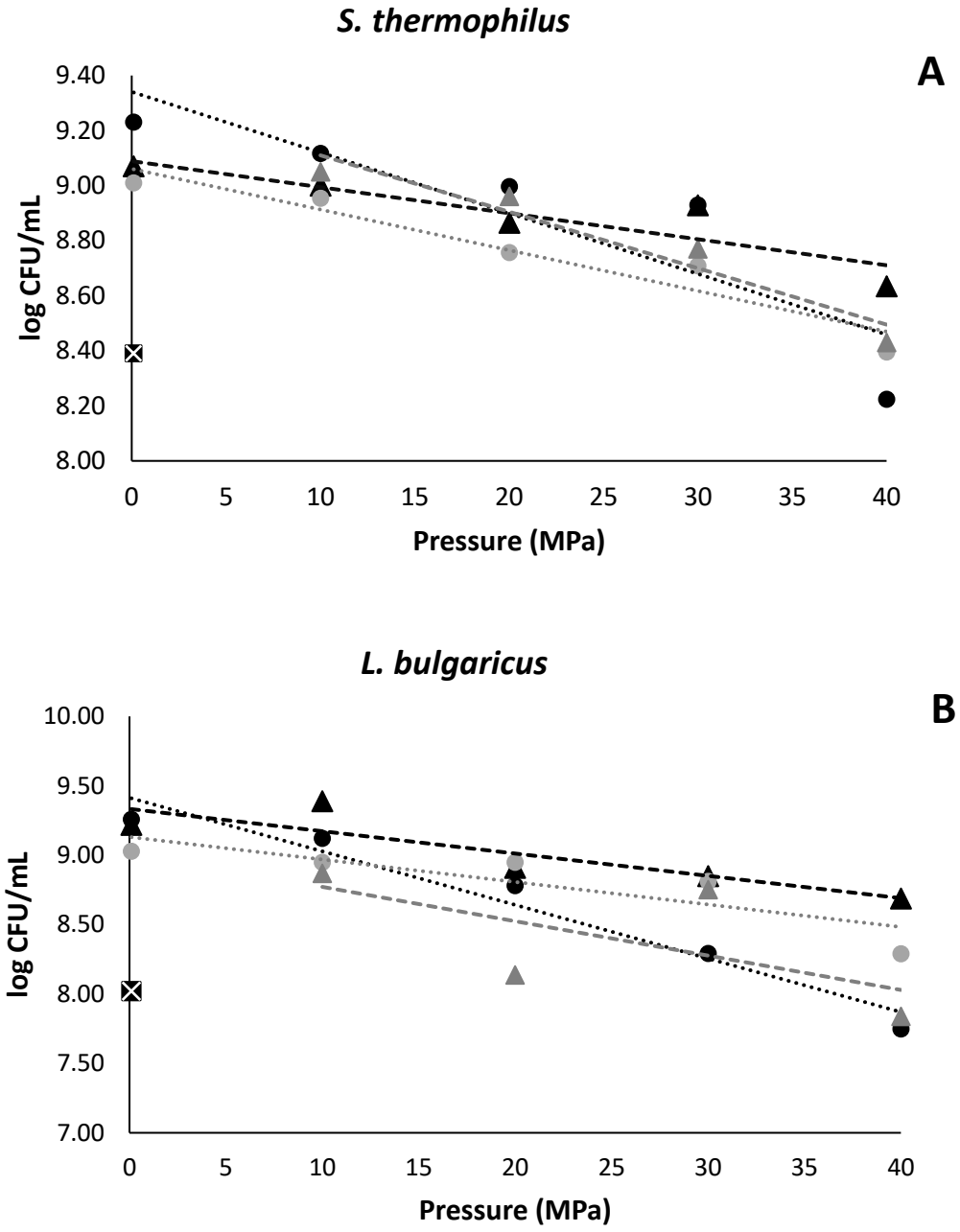


Figure II-2 - Lactic acid bacteria (Log CFU/mL) variation with fermentation pressure in yoghurts stored at 4°C fermented in different days (1st (●), 7th (▲) 15th (●) and 23rd (▲)): (A) *S. thermophilus* count and (B) *L. bulgaricus* count. Linear line equations: (A) $Y = -2.20 \times 10^{-2} x + 9.34$; $R^2 = 7.77 \times 10^{-1}$ (1st day); $Y = -9.46 \times 10^{-3} x + 9.09$; $R^2 = 7.98 \times 10^{-1}$ (7th day); $Y = -1.48 \times 10^{-2} x + 9.06$; $R^2 = 9.24 \times 10^{-1}$ (15th day); $Y = -2.05 \times 10^{-2} x + 9.32$; $R^2 = 9.30 \times 10^{-1}$ (23rd day); (B) $Y = -3.86 \times 10^{-2} x + 9.41$; $R^2 = 9.57 \times 10^{-1}$ (1st day); $Y = -1.61 \times 10^{-2} x + 9.33$; $R^2 = 7.86 \times 10^{-1}$ (7th day); $Y = -1.62 \times 10^{-2} x + 9.13$; $R^2 = 7.31 \times 10^{-1}$ (15th day); $Y = -2.48 \times 10^{-2} x + 9.02$; $R^2 = 4.21 \times 10^{-1}$ (23rd day). Note the different symbol (⊠) refers to an outlier of yoghurt fermented at 0.1 MPa in 23rd day of storage.

Table II-1 - Equations of pH linear variation between 1st and 23rd days of storage of yoghurts fermented at atmospheric pressure (0.1 MPa) and under pressure (10-40 MPa).

Pressure (MPa)	Slope	Intercept	R^2
0.1	-5.59×10^{-3}	4.48	0.858
10	-6.77×10^{-3}	4.54	0.940
20	-7.62×10^{-3}	4.56	0.892
30	-4.31×10^{-3}	4.53	0.919
40	-5.12×10^{-3}	4.55	0.912

Table II-2 - Yoghurt colour parameters evaluated for 23 days of storage.

Storage days vs Fermentation Pressure	1	7	15	23	Parameters
0.1 MPa	59.39 ± 0.59	58.21 ± 1.00	60.61 ± 1.21	59.38 ± 0.98^{B}	
10 MPa	59.73 ± 1.60	61.72 ± 1.96	59.86 ± 1.35	61.19 ± 0.71^{AB}	
20 MPa	57.86 ± 2.31	59.82 ± 0.68	59.79 ± 2.27	59.99 ± 0.91^{AB}	*L**
30 MPa	59.67 ± 1.14	59.89 ± 1.27	61.46 ± 1.61	61.41 ± 1.48^{A}	
40 MPa	56.23 ± 2.26	57.29 ± 0.30	57.83 ± 0.78	55.79 ± 1.57^{A}	
0.1 MPa	-1.04 ± 0.02	-0.98 ± 0.04	-1.13 ± 0.04	-1.05 ± 0.03	
10 MPa	-1.12 ± 0.05	-1.16 ± 0.07	-1.15 ± 0.06	-1.18 ± 0.05	
20 MPa	-1.10 ± 0.09	-1.15 ± 0.03	-1.23 ± 0.09	-1.23 ± 0.09	*a**
30 MPa	-1.18 ± 0.08	-1.23 ± 0.03	-1.21 ± 0.09	-1.21 ± 0.06	
40 MPa	-1.12 ± 0.16	-1.21 ± 0.09	-1.19 ± 0.06	-1.21 ± 0.07	
0.1 MPa	3.84 ± 0.27	3.44 ± 0.18	4.03 ± 0.12	3.76 ± 0.21	
10 MPa	4.30 ± 0.36	4.62 ± 0.37	4.42 ± 0.35	4.50 ± 0.07	
20 MPa	4.29 ± 0.58	4.65 ± 0.26	4.67 ± 0.52	4.75 ± 0.16	*b**
30 MPa	4.41 ± 0.50	4.61 ± 0.37	4.90 ± 0.21	4.82 ± 0.18	
40 MPa	4.39 ± 0.34	4.50 ± 0.51	4.34 ± 0.22	3.87 ± 0.25	
0.1 MPa		Control condition			
10 MPa	1.33 ± 0.68	3.74 ± 2.92	1.65 ± 0.98	2.01 ± 1.20	
20 MPa	2.44 ± 0.62	2.06 ± 0.85	1.86 ± 1.05	1.18 ± 0.22	ΔE
30 MPa	0.88 ± 0.40	2.30 ± 1.46	2.65 ± 1.56	2.86 ± 1.53	
40 MPa	3.98 ± 1.73	1.85 ± 0.36	2.81 ± 1.55	3.62 ± 1.25	

Different upper (A-B) case letters indicate statistical differences ($p < 0.05$) between pressures for the same storage period. Since colour parameters did not vary overall, to avoid repeat the same letter, only the cases where a statistical difference was verified ($p < 0.05$) have a letter (lower/upper case) to indicate this. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5% *a*nd all analyses were done in triplicate (n=3).

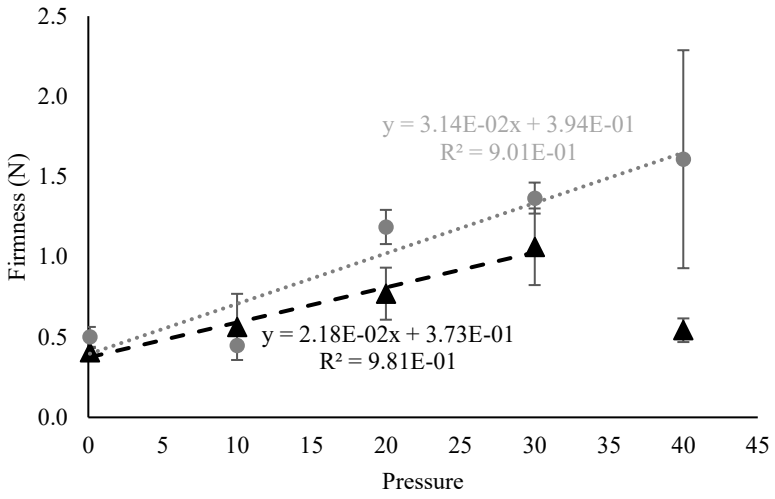


Figure II-3 - Firmness (N) variation with pressure for 1st ( ) and 15th ( ) day of storage. Linear line equations: $Y = 2.18 \times 10^{-2} x + 3.73 \times 10^{-1}$; $R^2 = 9.81 \times 10^{-1}$ (1st day) and $Y = 3.14 \times 10^{-2} x + 3.94 \times 10^{-1}$; $R^2 = 9.01 \times 10^{-1}$ (15th day). Error bars indicate standard deviation and all analysis were done in triplicate (n=3).

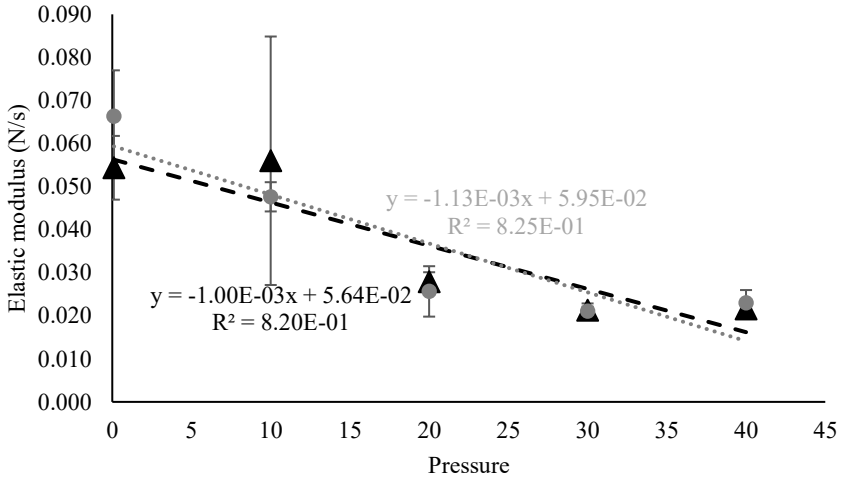


Figure II - 4 - Apparent elastic modulus (N/s) variation with pressure for 1st ( ) and 15th ( ) day of storage. Linear line equations: $Y = -1.00 \times 10^{-3} x + 5.64 \times 10^{-2}$; $R^2 = 8.02 \times 10^{-1}$ (1st day); $Y = -1.13 \times 10^{-3} x + 5.95 \times 10^{-2}$; $R^2 = 8.25 \times 10^{-1}$ (1st day); Error bars indicate standard deviation and all analysis were done in triplicate (n=3).

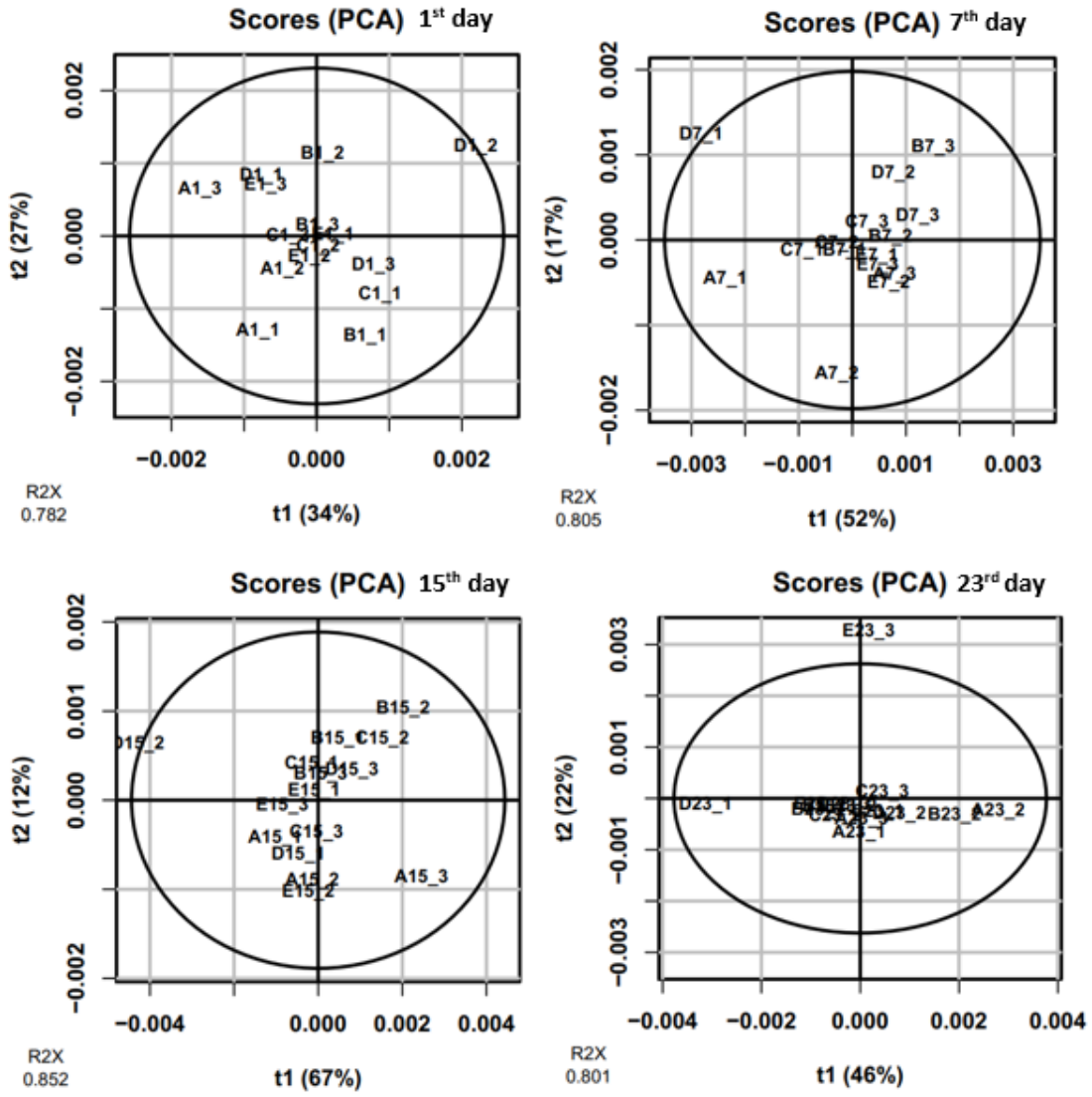


Figure II-5 - PCA scores plot of yoghurt produced under different conditions of pressure (0.1, 10, 20, 30 and 40 MPa) during different days of storage, obtained by 1D ^{1}H NMR. Legend of sample name code: Letters represent the pressure of fermentation A, B, C, D and E means 0.1, 10, 20, 30 and 40 MPa, the fist number at the right of letter mean the day of storage (1, 7, 15 or 23) and the second number represent the number of replica (1, 2 or 3).

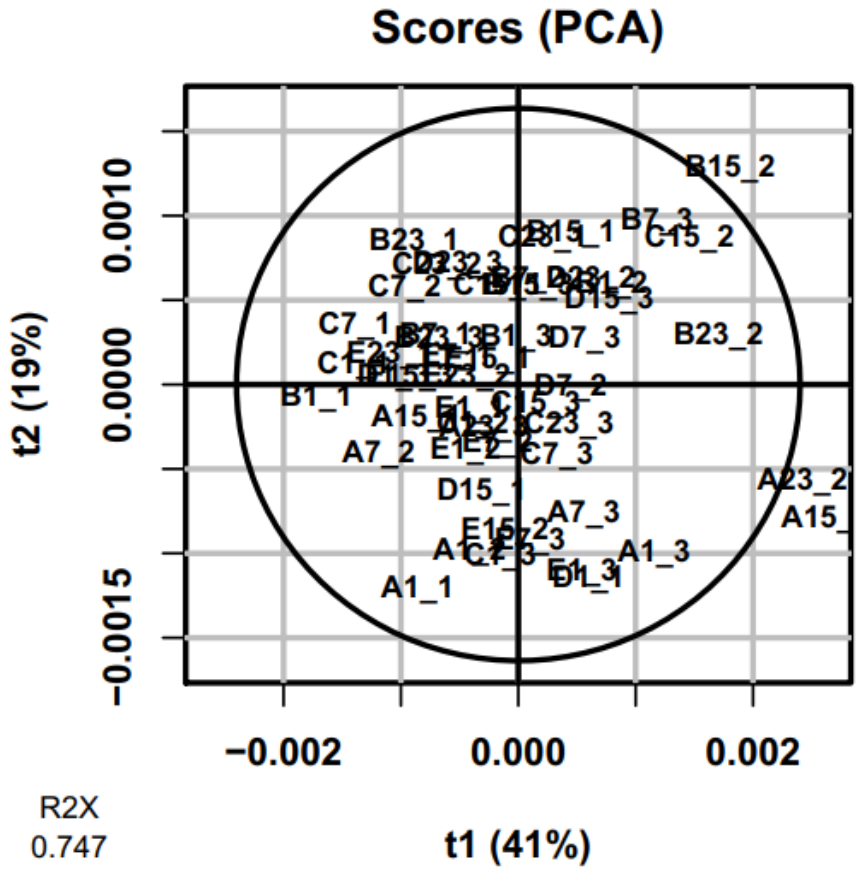


Figure II-6 - PCA scores plot of yoghurt produced under different conditions of pressure (0.1, 10, 20, 30 and 40 MPa) obtained by 1D ^{1}H NMR without the before identified outliers. Legend of sample name code: Letters represent the pressure of fermentation A to E means 0.1 to 40 MPa, the fist number at the right of letter mean the day of storage (1, 7, 15 or 23) and the second number represent the number of replica (1, 2 or 3).

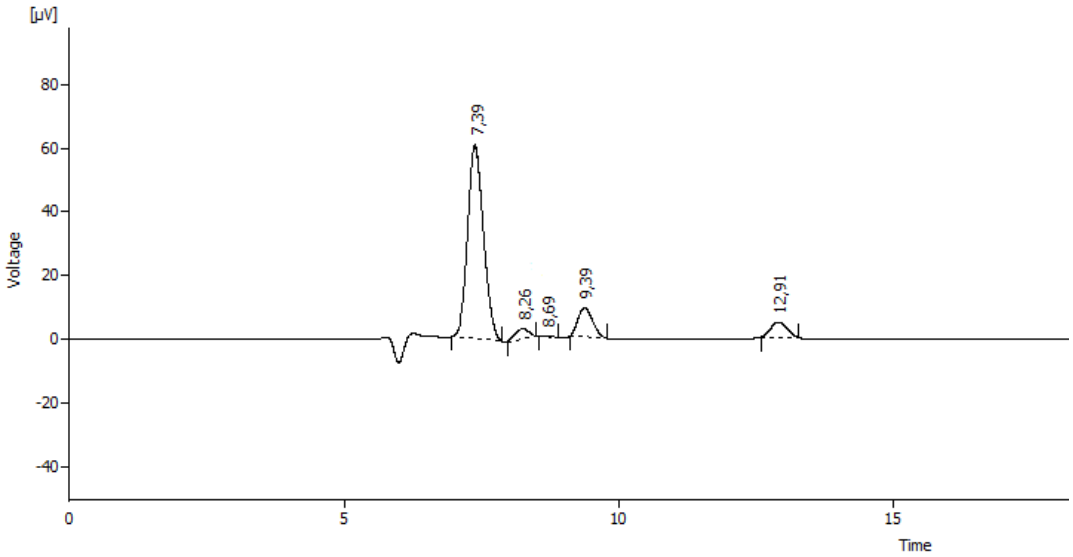


Figure II-7 - Chromatogram example of a yoghurt sample, obtained by high-performance liquid chromatography (HPLC) for sugars and organic acids assessment. **C**ompounds were identified by their retention time (min), namely lactose (7.39), citric acid (8.26), glucose (8.69), galactose (9.39) and lactic acid (12.91).

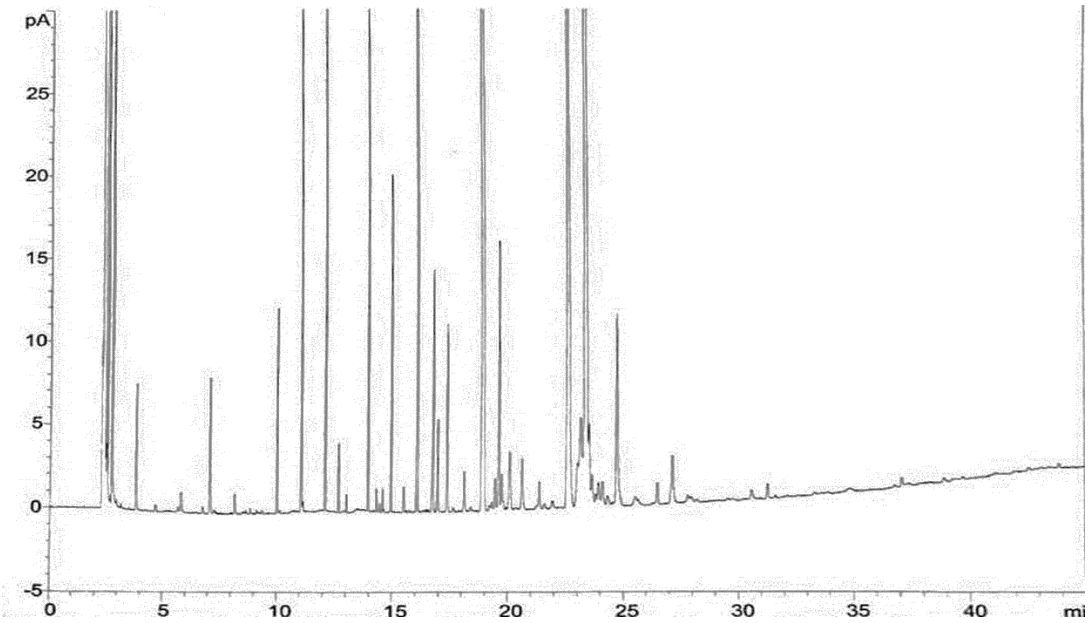


Figure II-8 – Yoghurt spectra example obtained by gas chromatography with a flame ionization detector (GC-FID) for total fatty acids (TFA) assessment.

Table II-3 – Fatty acid profile and content (µg/mg of yoghurt) of each yoghurt fermented under 0.1, 10, 20, 30 and 40 MPa at the first day of storage. Values are shown as mean ± standard deviation.

Day	1st day				
Pressure (MPa)	**0.1**	**10**	**20**	**30**	**40**
C4	201.1 ± 28.2^{B}	202.3 ± 17.7^{B}	177.6 ± 34.5^{B}	90.3 ± 15.8^{A}	90.7 ± 25.2^{A}
C6	235.0 ± 23.8^{B}	234.7 ± 21.7^{B}	213.9 ± 41.5^{B}	110.5 ± 18.8^{A}	106.5 ± 29.9^{A}
C8	226.7 ± 19.2^{B}	225.9 ± 21.8^{B}	202.9 ± 36.8^{B}	108.4 ± 18.8^{A}	104.8 ± 29.5^{A}
C10	637.0 ± 51.7^{B}	632.4 ± 57.1^{B}	571.7 ± 54.2^{B}	306.5 ± 58.2^{A}	286.6 ± 86.0^{A}
C10:1	61.7 ± 5.3^{B}	59.7 ± 5.9^{B}	53.9 ± 9.9^{B}	28.3 ± 5.1^{A}	28.2 ± 8.4^{A}
C12	913.0 ± 76.7^{B}	904.1 ± 79.4^{B}	812.9 ± 126.5^{B}	438.3 ± 51.3^{A}	402.6 ± 27.7^{A}
C13 i	23.6 ± 2.1^{B}	23.1 ± 2.5^{B}	20.9 ± 3.0^{B}	10.9 ± 2.1^{A}	10.4 ± 3.5^{A}
C13 ai	8.2 ± 1.3^{B}	7.7 ± 1.2^{B}	6.8 ± 1.2^{AB}	3.6 ± 1.1^{A}	3.8 ± 1.4^{A}
C12:1	24.2 ± 2.1^{B}	24.8 ± 1.9^{B}	21.5 ± 3.8^{B}	11.8 ± 2.3^{A}	10.7 ± 3.3^{A}
C14 i	428.2 ± 6.3^{A}	451.9 ± 22.1^{A}	454.9 ± 15.2^{A}	416.0 ± 27.8^{A}	424.7 ± 43.1^{A}
C14	30.8 ± 3.0^{C}	22.8 ± 1.6B^{C}	20.0 ± 3.1^{B}	10.8 ± 2.4^{A}	13.5 ± 4.7^{AB}
C14:1	3143.0 ± 276.8^{B}	2952.3 ± 245.8^{B}	2650.6 ± 373.9^{B}	1432.3 ± 304.2^{A}	1360.3 ± 436.2^{A}
C15	377.8 ± 31.4^{B}	350.3 ± 28.4^{B}	312.8 ± 46.8^{B}	175.1 ± 33.6^{A}	171.9 ± 48.0^{A}
C15:1	151.9 ± 4.3^{B}	133.2 ± 11.4^{B}	118.4 ± 15.5^{B}	63.8 ± 13.5^{A}	65.5 ± 20.2^{A}
C16	301.7 ± 25.9^{B}	281.7 ± 23.0^{B}	252.9 ± 34.9^{B}	136.6 ± 17.5^{A}	129.5 ± 8.0^{A}
C16:1 (c7)	9473.0 ± 865.7^{B}	9041.6 ± 733.7^{B}	8066.3 ± 1048.1^{B}	4391.7 ± 955.4^{A}	4054.9 ± 1314.7^{A}
C16:1 (c9)	64.6 ± 10.1^{B}	65.4 ± 9.5^{B}	56.2 ± 12.7^{B}	28.6 ± 8.3^{A}	24.9 ± 8.1^{A}
C17 i	501.5 ± 8.7^{B}	486.1 ± 44.3^{B}	435.6 ± 65.4^{B}	233.0 ± 51.2^{A}	212.3 ± 68.9^{A}
C17 ai	90.6 ± 13.3^{B}	82.1 ± 9.6^{B}	73.2 ± 11.7^{B}	38.7 ± 10.4^{A}	32.7 ± 12.7^{A}
C17	185.2 ± 21.6^{B}	165.3 ± 0.2^{B}	142.1 ± 16.4^{B}	80.1 ± 19.3^{A}	79.6 ± 26.1^{A}
C17:1	150.8 ± 19.8^{B}	137.7 ± 11.2^{B}	121.6 ± 15.7^{B}	65.9 ± 16.6^{A}	64.5 ± 23.4^{A}
C18	69.7 ± 8.7^{B}	66.6 ± 5.2^{B}	57.3 ± 9.2^{B}	29.8 ± 8.2^{A}	29.2 ± 10.2^{A}
C18:1 (t12)	2779.8 ± 267.2^{B}	2520.5 ± 197.4^{B}	2242.7 ± 261.7^{B}	1237.7 ± 272.5^{A}	1177.5 ± 374.6^{A}
C18:1 (c9)	399.6 ± 44.5^{B}	436.2 ± 21.6^{B}	392.0 ± 46.8^{B}	208.3 ± 44.7^{A}	173.3 ± 52.9^{A}

C18:1 (t15)	6169.5	±	574.6^{B}	5751.8	±	468.7^{B}	5128.3	±	649.5^{B}	2796.9	±	607.4^{A}	2636.1	±	844.4^{A}
C18:1 (c11)	235.7	±	31.8^{B}	223.4	±	18.3^{B}	195.2	±	27.5^{B}	107.2	±	24.8^{A}	101.2	±	29.1^{A}
C18:2	110.9	±	5.3^{B}	127.0	±	9.0^{B}	106.3	±	17.7^{B}	56.4	±	14.8^{A}	48.0	±	14.7^{A}
C18:3	591.7	±	65.7^{B}	691.2	±	60.6^{B}	644.7	±	23.4^{B}	344.8	±	75.4^{A}	272.1	±	82.5^{A}
C20	54.5	±	6.8^{B}	78.9	±	10.2^{B}	74.1	±	13.1^{AB}	40.9	±	8.9^{A}	28.2	±	9.0^{A}
CLA	201.3	±	20.6^{B}	199.2	±	19.3^{B}	169.6	±	12.4^{B}	100.3	±	16.2^{A}	93.0	±	24.6^{A}
C20:4	79.3	±	6.0^{B}	66.8	±	17.3^{B}	55.9	±	11.5^{AB}	31.3	±	8.8^{A}	30.4	±	8.6^{A}
C22	41.2	±	8.6^{B}	35.5	±	4.4^{B}	28.3	±	4.2^{AB}	15.8	±	2.9^{A}	13.2	±	3.4^{A}
C24	43.7	±	9.9^{A}	49.4	±	7.0^{A}	42.1	±	7.9^{A}	23.4	±	5.1^{A}	20.7	±	5.9^{A}

Different lower upper (A-C) case letters indicate statistical differences ($p < 0.05$) between pressures, respectively. No statistical difference was found along the storage period. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Table II-4 – Fatty acid profile and content (μg/mg of yoghurt) of each yoghurt fermented under 0.1, 10, 20, 30 and 40 MPa at the 23rd day of storage. Values are shown as mean ± standard deviation.

Day	**23rd day**														
Pressure (MPa)	**0.1**			**10**			**20**			**30**			**40**		
C4	230.6	±	8.9^{C}	183.4	±	13.0^{B}	129.0	±	17.7^{A}	108.1	±	11.7^{A}	102.2	±	11.5^{A}
C6	274.3	±	10.5^{C}	220.5	±	15.2^{B}	151.1	±	11.6^{A}	130.7	±	13.2^{A}	121.0	±	13.4^{A}
C8	258.9	±	8.5^{C}	211.8	±	14.4^{B}	146.5	±	10.0^{A}	129.1	±	11.7^{A}	117.5	±	12.5^{A}
C10	701.3	±	22.0^{C}	580.7	±	32.3^{B}	409.1	±	48.3^{A}	362.0	±	34.4^{A}	322.2	±	36.5^{A}
C10:1	69.8	±	2.7^{C}	56.0	±	4.5^{B}	38.7	±	4.4^{A}	34.2	±	3.5^{A}	32.0	±	3.4^{A}
C12	970.2	±	29.7^{B}	824.3	±	41.8^{B}	581.5	±	59.5^{A}	521.6	±	6.7^{A}	450.8	±	52.7^{A}
C13 i	25.1	±	0.8^{B}	21.0	±	1.3^{B}	14.5	±	2.0^{A}	12.9	±	1.6^{A}	11.9	±	1.7^{A}
C13 ai	9.3	±	0.6^{C}	6.6	±	0.6^{B}	4.8	±	0.6^{AB}	4.1	±	0.9^{A}	4.6	±	0.6^{AB}
C12:1	25.9	±	1.0^{B}	21.4	±	1.8^{B}	14.9	±	1.6^{AB}	14.0	±	1.4^{AB}	11.7	±	1.2^{A}
C14 i	414.3	±	12.0^{B}	421.2	±	18.5^{A}	427.1	±	49.1^{A}	429.2	±	32.2^{A}	408.8	±	15.7^{A}
C14	33.3	±	2.0^{B}	20.5	±	1.2^{A}	13.9	±	2.0^{A}	12.2	±	1.1^{A}	15.1	±	1.9^{A}

C14:1	3263.8	±	109.0^{C}	2625.8	±	153.0^{B}	1889.7	±	243.9^{A}	1679.3	±	170.4^{A}	1520.1	±	176.2^{A}
C15	397.4	±	12.9^{C}	314.2	±	18.0^{B}	226.7	±	28.0^{A}	201.8	±	20.8^{A}	187.5	±	20.9^{A}
C15:1	155.8	±	1.9^{C}	118.4	±	7.3^{B}	83.0	±	11.2^{A}	74.4	±	6.9^{A}	72.3	±	8.1^{A}
C16	311.1	±	10.4^{C}	248.8	±	16.1^{B}	180.3	±	23.1^{A}	159.1	±	7.1^{A}	145.2	±	7.1^{A}
C16:1 (c7)	9714.7	±	343.7^{B}	7932.2	±	521.3^{B}	5771.9	±	775.1^{A}	5134.0	±	548.3^{A}	4534.2	±	516.7^{A}
C16:1 (c9)	63.3	±	3.3^{B}	55.9	±	6.6^{B}	33.7	±	5.0^{A}	33.2	±	5.3^{A}	27.2	±	2.8^{A}
C17 i	517.3	±	14.7^{B}	430.4	±	24.1^{B}	303.3	±	38.6^{A}	272.9	±	30.4^{A}	238.5	±	28.0^{A}
C17 ai	85.4	±	1.4^{B}	71.5	±	3.6^{B}	48.3	±	6.5^{A}	45.1	±	6.0^{A}	38.5	±	4.9^{A}
C17	184.3	±	7.8^{C}	146.9	±	11.7^{B}	103.1	±	13.5^{A}	94.0	±	11.7^{A}	88.4	±	9.3^{A}
C17:1	152.3	±	6.8^{C}	119.6	±	9.5^{B}	86.4	±	11.9^{A}	79.5	±	10.5^{A}	68.9	±	7.2^{A}
C18	74.1	±	4.6^{C}	56.9	±	2.3^{B}	40.2	±	5.0^{A}	35.7	±	3.8^{A}	32.0	±	7.0^{A}
C18:1 (t12)	2817.7	±	99.0^{C}	2184.6	±	156.7^{B}	1602.1	±	218.9^{A}	1432.4	±	154.6^{A}	1323.4	±	143.7^{A}
C18:1 (c9)	424.0	±	10.6^{B}	377.4	±	26.6^{B}	273.1	±	29.3^{A}	243.0	±	24.5^{A}	196.3	±	22.3^{A}
C18:1 (t15)	6328.1	±	218.0^{C}	5027.6	±	332.6^{B}	3644.9	±	469.4^{A}	3254.6	±	344.4^{A}	2956.4	±	329.0^{A}
C18:1 (c11)	247.2	±	7.9^{C}	194.5	±	20.6^{B}	133.3	±	19.0^{A}	120.7	±	6.2^{A}	112.0	±	15.1^{A}
C18:2	113.2	±	4.8^{B}	109.5	±	9.4^{B}	73.3	±	6.8^{A}	66.9	±	7.0^{A}	52.9	±	5.4^{A}
C18:3	623.7	±	12.4^{C}	631.3	±	41.2^{C}	453.3	±	49.1^{B}	410.3	±	42.9^{AB}	301.0	±	32.9^{A}
C20	71.1	±	5.1^{BC}	78.5	±	6.5^{C}	54.4	±	5.8^{B}	47.7	±	5.2^{AB}	32.4	±	3.8^{A}
CLA	216.9	±	8.8^{B}	182.8	±	17.4^{B}	128.1	±	11.9^{A}	116.7	±	15.2^{A}	114.6	±	13.9^{A}
C20:4	70.3	±	6.8^{B}	57.1	±	9.5^{B}	34.3	±	2.1^{A}	38.5	±	2.2^{A}	32.6	±	3.4^{A}
C22	41.0	±	6.8^{B}	31.0	±	1.6^{B}	20.2	±	2.4^{AB}	19.5	±	1.9^{AB}	14.8	±	1.6^{A}
C24	39.0	±	20.6^{A}	44.6	±	2.4^{A}	30.4	±	4.0^{A}	28.4	±	3.7^{A}	23.5	±	3.1^{A}

Different lower upper (A-C) case letters indicate statistical differences ($p < 0.05$) between pressures, respectively. No statistical difference was found along the storage period. The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences (HSD) test, at a significance of 5 %. Error bars indicate standard deviation and all analyses were done in triplicate (n=3).

Table II – 3 – Fatty acid profile and content (μg/mg of yoghurt) per group of each yoghurt fermented under 0.1, 10, 20, 30 and 40 MPa at the 23rd day of storage.

Day	**1st**											
Pressure (MPa)	**0.1**			**10**			**20**			**30**			**40**		
TFA	28006.5	±	2547.1	26731.4	±	2189.0	23923.2	±	3055.6	13174.2	±	2723.4	12301.5	±	3689.1
SCFA+ SFA	4369.4	±	347.1	4300.7	±	357.4	3900.0	±	525.6	2268.9	±	353.5	2160.7	±	443.3
MUFA	22653.9	±	2102.3	21346.5	±	1725.4	19046.7	±	2465.1	10372.6	±	2254.7	9697.2	±	3115.3
PUFA	983.2	±	97.6	1084.2	±	106.2	976.5	±	65.0	532.8	±	115.2	443.6	±	130.5
Total *n*-3	591.7	±	65.7	691.2	±	60.6	644.7	±	23.4	344.8	±	75.4	272.1	±	82.5
Total *n*-6	190.2	±	11.3	193.8	±	26.3	162.2	±	29.2	87.7	±	23.6	78.4	±	23.3
cis	14169.3	±	1319.9	13674.8	±	1109.8	12232.9	±	1565.8	6635.2	±	1444.2	6099.2	±	1961.7
trans	9011.0	±	847.1	8331.9	±	672.0	7424.9	±	921.0	4063.0	±	885.0	3841.8	±	1227.4
Day	**23rd**											
Pressure (MPa)	**0.1**			**10**			**20**			**30**			**40**		
TFA	28924.8	±	1016.5	23606.7	±	1542.5	17145.2	±	2187.3	15346.0	±	1547.3	13710.8	±	1513.5
SCFA+ SFA	4638.1	±	179.6	3912.7	±	224.7	2884.4	±	327.8	2614.2	±	204.0	2355.0	±	232.2
MUFA	23262.6	±	804.1	18713.4	±	1240.3	13571.8	±	1789.6	12099.4	±	1276.0	10854.5	±	1225.8
PUFA	1024.1	±	32.8	980.6	±	77.5	689.0	±	69.9	632.5	±	67.3	501.2	±	55.6
Total *n*-3	623.7	±	12.4	631.3	±	41.2	453.3	±	49.1	410.3	±	42.9	301.0	±	32.9
Total *n*-6	183.5	±	11.6	166.6	±	18.9	107.6	±	8.9	105.4	±	9.2	85.6	±	8.8
cis	14602.1	±	498.6	12046.1	±	788.0	8714.6	±	1140.0	7767.0	±	815.1	6812.6	±	778.6
trans	9215.6	±	319.7	7268.2	±	493.8	5285.8	±	692.8	4721.2	±	502.5	4311.9	±	476.1

Total fatty acids (TFA); short-chain fatty acids and short fatty acids (SCFA+ SFA) monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); Total fatty acids omega 3 (Total *n*-3); Total fatty acids omega 6 (Total *n*-6); Total cis unsaturated fatty acid (cis); Total trans unsaturated fatty acid (trans);

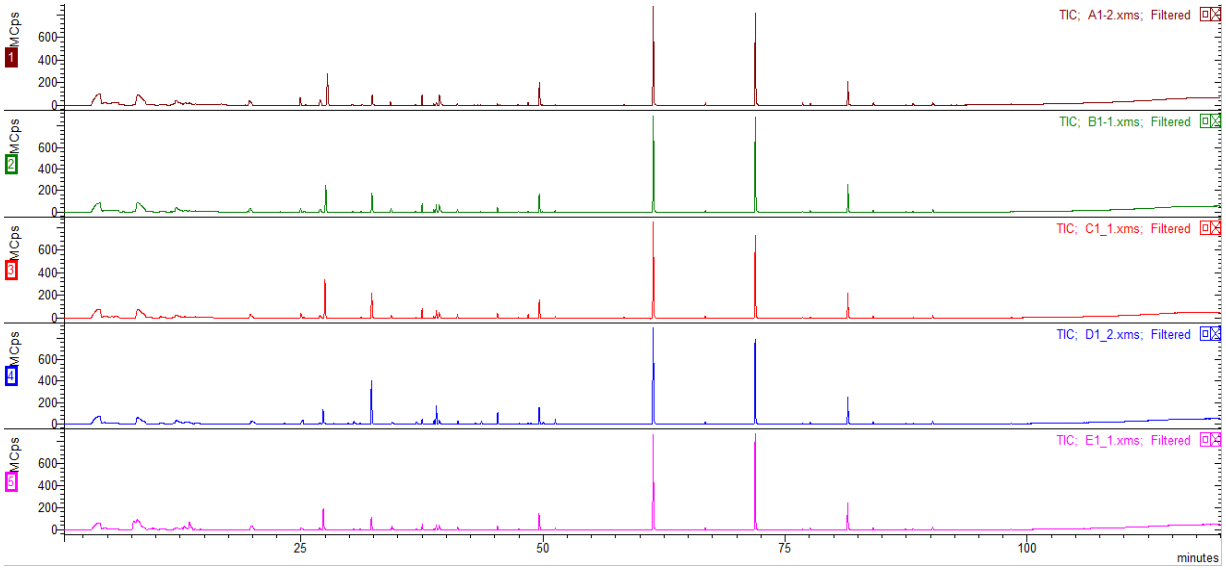


Figure II-9 - Chromatogram of yoghurt produced under different pressures 0.1 (A) (brown spectra), 10 (B) (green spectra), 20 (C) (red spectra), 30 (D) (blue spectra) and 40 (E) (pink spectra) MPa at 43ºC, for the first day of storage.